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UNIVERSITY OF ALBERTA

STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE FinOP FERTILITY INHIBITION SYSTEM

BY

©

TIM VAN BIESEN

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

DEPARTMENT OF MICROBIOLOGY

Edmonton, Alberta
Spring, 1994

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Date: Feb 11, 1994

To my parents

Jacques and *Coby*

for their love, strength and inspiration

Abstract

Most of the genes required for the conjugal transfer of DNA among bacteria are encoded by the 30 kb transfer (*tra*) operon of F-like conjugative plasmids. Expression of the *tra* operon is positively regulated by the plasmid-encoded TraJ transcriptional activator which, in turn, is negatively regulated by the FinOP (fertility inhibition) system. The FinOP system consists of a 78 base antisense RNA, FinP, and a 21.2 kDa protein, FinO, which act in concert to inhibit TraJ translation.

An analysis of the two proposed alleles of *finO* revealed that the level of FinO expression is increased in some plasmids by the presence of an upstream co-transcribed region of DNA, *orf286*. This *orf286-finO* transcript has an extended functional half-life *in vivo*, resulting in an increased level of FinO translation and a subsequent increase in the efficiency of fertility inhibition.

The *in vitro* secondary structures of FinP RNA and its target, *traJ* mRNA, were determined using structure-specific ribonucleases. The results revealed that the two RNAs adopt complementary double stem-loop structures, indicating that they have the potential to form a duplex with each other. The apparent second order rate constant of FinP-*traJ* mRNA duplex formation was found to be similar to other previously characterized naturally occurring antisense RNA systems. Primer extension analysis and Northern blotting of FinP and *traJ* mRNA revealed that FinO protects FinP from degradation and the FinOP complex reduces the steady-state levels of *traJ* mRNA by more than 50-fold.

FinO protein was purified as a fusion with glutathione-S-transferase using a glutathione affinity resin. The GST-FinO fusion bound directly to the second stem-loop domain of both FinP and *traJ* mRNA *in vitro*. Analysis of the kinetics of FinP-*traJ*

mRNA duplex formation with and without FinO revealed that FinO increases the rate of duplex formation by a factor of 5.

These results, combined with the characterization of the *fisO* class of FinP mutants, indicate that FinO is an unusual multifunctional protein which is involved in both the protection of FinP from RNase-mediated degradation and the catalysis of the formation of the FinP-*traJ* mRNA duplex. A model of FinOP-mediated inhibition of TraJ expression is proposed.

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The completion of the work presented in this thesis would not have been possible without the excellent supervision of Dr. Laura S. Frost. Her constant enthusiasm for science and her youthful optimism provided me with the motivation and the confidence required for the completion of such a complex project. I will always be indebted to Laura for her belief in me and her willingness to allow me to participate in international meetings, workshops and collaborations. In addition to being my mentor, Laura will always remain my very good friend.

I am also indebted to Dr. William Paranchych who, as a friend and as chairman of the department, actively supported my endeavors as a graduate student and taught me how the responsibilities of administration can be both rewarding and frustrating.

The months of November and December, 1992 were spent in Uppsala, Sweden as a guest of Drs. Kurt Nordström and Gerhart Wagner. Along with Fredrik Söderbom, the four of us carried out a very productive collaboration, the result of which is described in Chapter 3 of this thesis. I appreciate their patience and hospitality and I hope to one day return for a burger and a beer at Norlands Student Nation.

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Finally, I am deeply indebted to my parents, to whom this thesis is dedicated, and to Vida Foubister, to whom my future is dedicated.

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Abbreviations

ATP	adenine; adenosine triphosphate
BSA	bovine serum albumin
C	cytidine
cAMP	cyclic adenosine monophosphate
CRP	cAMP receptor protein
Δ	deletion
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
F	F conjugative plasmid
fin	fertility inhibition
G	guanidine
inc	incompatibility
IPTG	isopropyl -β-D-thiogalactopyranoside
IS	insertion sequence
kb; kbp	kilobases; kilo base pairs
kDa	kilo-Daltons
M	molar concentration
min; m	minutes
orf	open reading frame
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
RNase	ribonuclease
SDS	sodium dodecyl sulphate
sec; s	seconds
T	thymidine
TCS	trypticase soy broth
Tn	transpsoson
UTP	uridine triphosphate

Chapter 1

Introduction

The ability of bacteria to transfer genetic information horizontally allows them to adapt to changes in the environment in a rapid and specific manner. This phenomenon is largely responsible for the spread of antibiotic resistance among pathogenic bacteria, resulting in continuing efforts to develop new antibiotics and to regulate the distribution of those already in use. Gene transfer also allows bacteria to acquire such functions as the metabolism of complex organic compounds and the synthesis of toxins.

There are several mechanisms by which cells might acquire additional genetic information from neighboring but unrelated populations. *Natural transformation* is the process by which cells pick up and express exogenous DNA. *Transduction* involves the transfer of DNA between cells via bacteriophages which can be propagated only within bacterial hosts. *Transposable elements* are discrete DNA segments which are highly mobile in the genome, without the requirement of the extensive DNA homology required for classical recombination. Finally, *bacterial conjugation* is the process by which a single strand of DNA is transferred from a donor to a recipient cell via a direct physical association. It is the regulation of bacterial conjugation that is the subject of this thesis.

A. Bacterial Conjugation

The discovery of bacterial conjugation by Lederberg in 1946 is viewed by many as the dawn of modern molecular biology (Lederberg and Tatum, 1946). This fortuitous event, for it required that several unlikely incidents occur at once, gave rise to basic concepts such as the structure of the bacterial chromosome (Jacob and Wollman, 1961). The exact mechanism of the conjugal transfer of DNA has received renewed attention since the development of recombinant DNA technology and is the subject of several recent reviews (Frost, 1992; Frost *et al*, 1993).

Conjugative plasmids are classified according to their ability to co-exist in a bacterial cell. Two or more plasmids unable to replicate in the same cell are said to belong to the same incompatibility (Inc) group. This incompatibility is the result of similarities at the level of plasmid replication and partitioning (Austin and Nordström, 1990). There are greater than 20 Inc groups, the best studied of which are the IncP and IncF groups. The IncP group consists of "promiscuous" plasmids, so named due to their ability to replicate and transfer among a wide variety of bacterial species. The IncF group is best represented by the F plasmid itself and also contains the "R" plasmids (for resistance) which are most directly responsible for the transmission of antibiotic resistance determinants (Willetts and Maule, 1986). This thesis deals only with members of the IncF group of plasmids, F being the commonly used example.

The process of bacterial conjugation (Fig. 1a) involves a cell surface appendage, the pilus, which directly recognizes and binds to the surface of a recipient cell, that is, a cell that does not carry a plasmid that is incompatible with that found in the donor cell. After this initial event, the cells are brought into intimate contact with each other, presumably by retraction of the pilus into the donor cell, and a stable mating pair is formed. The transfer of DNA is activated and a single strand of the plasmid is transmitted to the recipient cell. The single strands of DNA are replicated in both the donor and recipient cells, resulting in a complete copy of the plasmid in both cells. For the 100 kb F plasmid, the entire conjugative event lasts no more than 5 minutes at 37°C.

All of the genes required for the transfer of F-plasmid DNA are encoded by a 33 kb operon known as the transfer (*tra*) operon (Fig. 1b). Some of the functions encoded by this operon include pilus synthesis and assembly, surface exclusion, mating pair stabilization, nicking, unwinding and transport of the DNA. Recently, the entire sequence of the F *tra* operon has been made available and a comprehensive analysis of all the known gene products has been presented (Frost *et al*, 1994). It is interesting to note that among the more than 30 gene products, there is very little detectable similarity

Figure 1a: The process of bacterial conjugation. A schematic diagram showing the basic steps in the conjugative transfer of bacterial DNA.

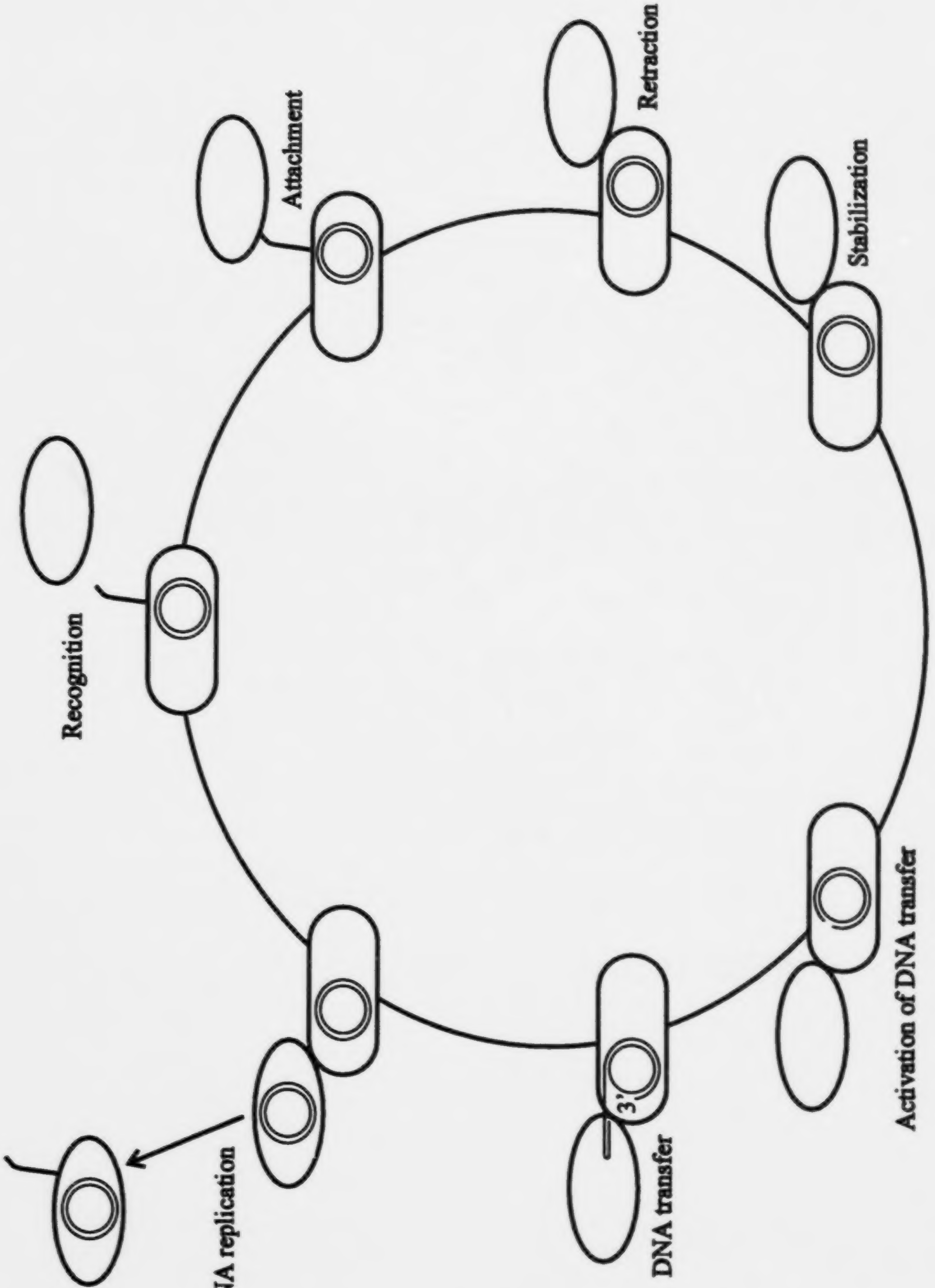
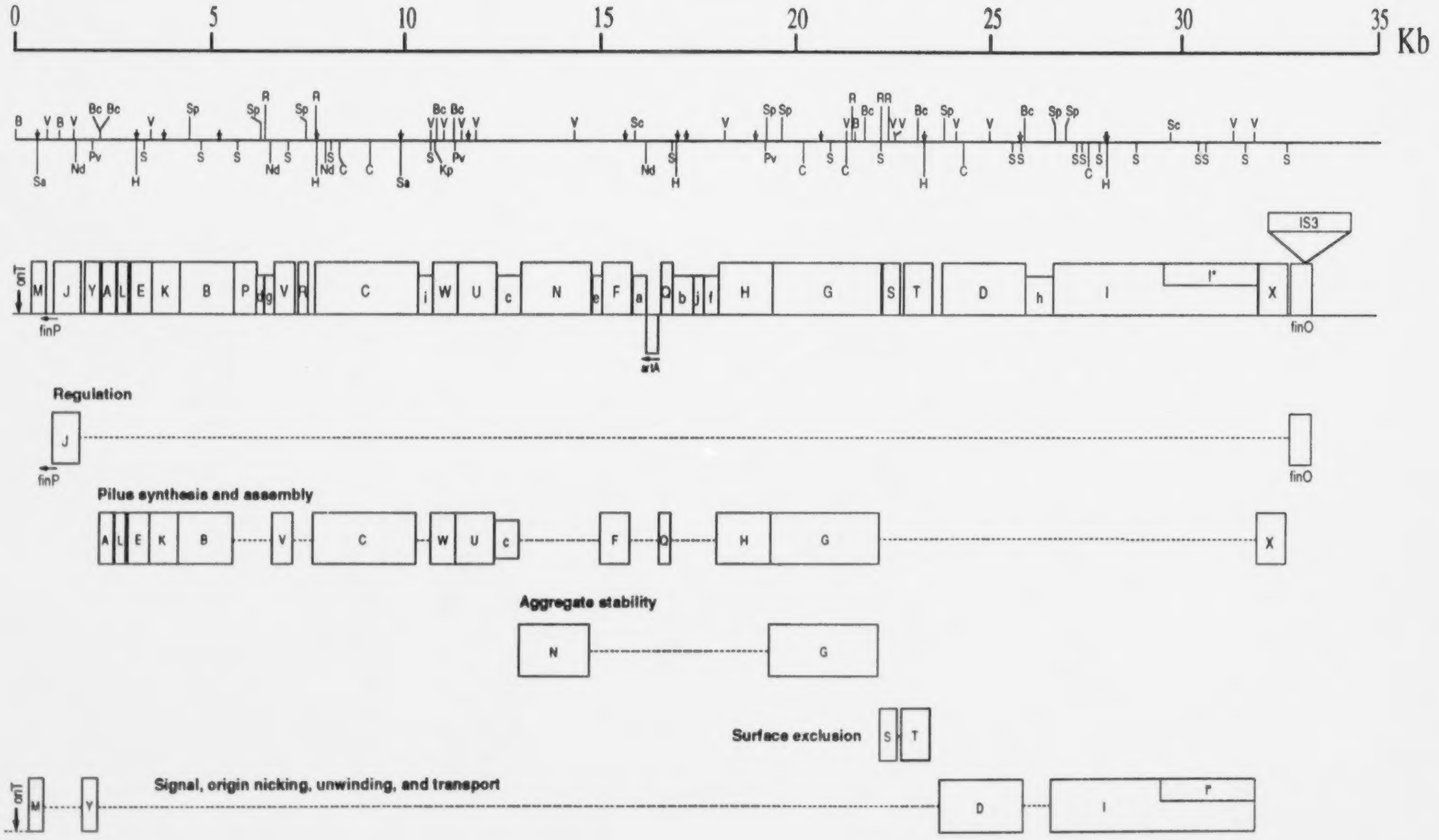

Recognition
Attachment
Retraction
Stabilization
Activation of DNA transfer
DNA transfer
3'
DNA replication

Figure 1b: A comprehensive map of the F transfer region. The top line shows the length in kilobases. The second line indicates the position of restriction sites: B=*Bgl*II, Sa=*Sal*I, V=*Eco*RV, Nd=*Nde*I, Bc=*Bcl*I, H=*Hpa*I, S=*Sma*I, Sp=*Sph*I, C=*Cla*I, R=*Eco*RI, Kp=*Kpn*I, arrows=*Hinc*II. The third lines shows the gene products: uppercase letters indicate *tra* genes and lower case letters indicate *trb* genes. The last five lines indicate the functions of the *tra* genes identified to date.


0
5
10
15
20
25
30
35
Kb
oriT
finP
M J Y A L E K B P d g V R C i W U c N e F a Q b j f H G S T D h I I* X
IS3
finO
artA
Regulation
J
finP
finO
Pilus synthesis and assembly
A L E K B V C W U c F Q H G X
Aggregate stability
N G
Surface exclusion
S T
Signal, origin nicking, unwinding, and transport
oriT
M Y D I I*

to other sequences in the databanks.

In addition to those genes which form the *tra* operon, there are a number of other genes which are associated with the regulation of DNA transfer. For example, *traM* encodes an autoregulatory protein (TraM) which is thought to be involved in the signaling event, that is, the point at which transfer of the DNA should begin (DiLaurenzio *et al*, 1992). *traY* is the first gene of the *tra* operon and contributes to the regulation of the expression of both *traM* and the entire *tra* operon. The *traJ*, *finP*, and *finO* genes are directly involved in regulating the expression of the *tra* operon and are discussed in detail below.

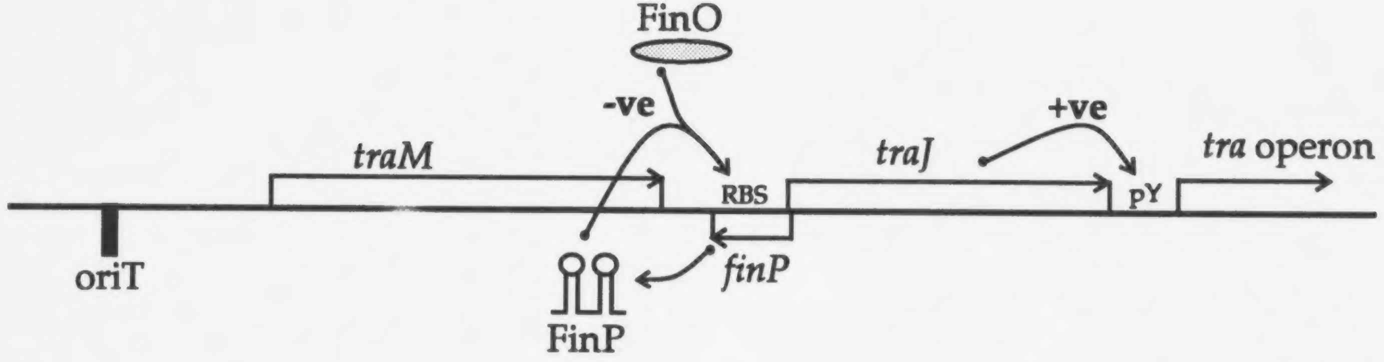
B. Fertility Inhibition

The fertility inhibition system of IncF plasmids consists of two components, an antisense RNA molecule, FinP, and a second gene, *finO*, the nature of which, prior to the work presented herein, was unknown. These two gene products act in concert to inhibit the expression of TraJ, the transcriptional activator of the *tra* operon. This process is shown schematically in figure 1c and is described in detail below.

I. TraJ - A Transcriptional Activator

TraJ protein (27 kDa), the product of the *traJ* gene, is a cytoplasmic protein that activates transcription of the *tra* operon from the pY promoter (Cuozzo and Silverman, 1986; Willetts, 1977). Although mutational analysis has revealed the pY promoter to be the site of action, direct binding of the protein to the DNA has not been demonstrated. As a result, the mechanism by which TraJ activates transcription is unknown.

Figure 1c: The *tra* regulatory region. The functions of the three main regulatory gene products, TraJ, FinP and FinO are described in the text. RBS = Ribosome Binding Site, oriT = origin of DNA transfer. -ve and +ve refer to negative and positive regulatory effects, respectively.


FinO
-ve
+ve
traM
RBS
traJ
pY
tra operon
oriT
finP
FinP

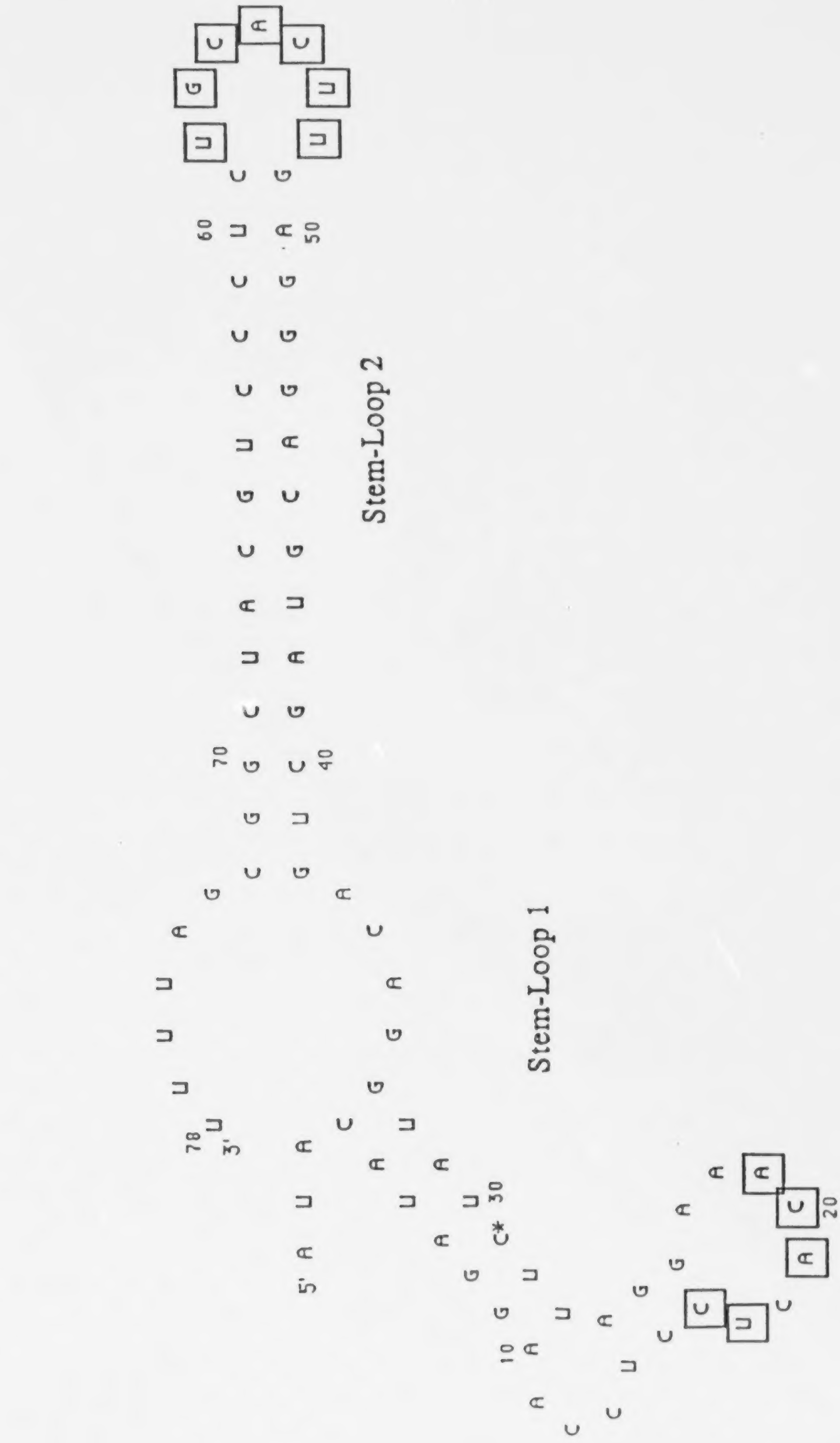
Gaudin and Silverman (1993) have proposed that the pY promoter is held in a locally relaxed conformation as a result of either *traJ* transcription (which occurs directly upstream) or an as yet unidentified nucleoprotein complex. In their model, TraJ disrupts this conformation and restores normal levels of supercoiling, thereby allowing transcription to proceed from this promoter. Among the six characterized alleles of *traJ* (Willetts and Maule, 1986; DiLaurenzio *et al*, 1991; Graus-Goldner *et al*, 1990), there is surprisingly little similarity. Most of the homology resides within a domain which resembles a helix-turn-helix DNA binding motif (Frost *et al*, 1994).

II. FinP - An Antisense RNA

The *finP* gene is located in the same region as the 5'-end of the *traJ* gene but it is transcribed in the opposite orientation. As such, it is not under the control of the pY promoter and has been shown to be transcribed constitutively from its own promoter (Dempsey, 1987). The product of the *finP* gene is a 78 base RNA molecule which is complementary to the 5'-untranslated region of the *traJ* mRNA. Six alleles of *finP* have been identified (Willetts and Maule, 1986) and all of these have been cloned and sequenced (Fee and Dempsey, 1986; Finlay *et al*, 1986). As is the case with the *traJ* alleles, all of the *finP* alleles characterized thus far are plasmid specific (Willetts and Maule, 1986). FinP is thought to form a stable RNA-RNA duplex with the *traJ* mRNA, thereby occluding the *traJ* ribosome binding site and preventing TraJ synthesis. Note that the inhibitory effect of FinP occurs only in the presence of the second fertility inhibition gene, *finO* (Willetts, 1977).

FinP has been predicted to fold into a double stem-loop structure that is characteristic of other antisense RNA molecules (Koraimann *et al*, 1991; Finlay *et al*, 1986) (Fig. 1d). Interestingly, the bulk of the sequence variations among the *finP* alleles is concentrated in the proposed loop regions of the molecule whereas the inverted repeats which give rise to the stem sequences are highly conserved (Finlay *et al*, 1986). Also, site-specific

Figure 1d: The predicted structure of FinP RNA. The structure of FinP RNA as predicted by a computer algorithm (Zuker and Stiegler, 1981). Figure courtesy of Stuart Lee (1992).


Stem-Loop 1
Stem-Loop 2
C*

mutational analysis of FinP has revealed that the most deleterious mutations are located in the loop regions, suggesting that the loops are responsible for the observed plasmid specificity (Koraimann *et al*, 1991; Finlay *et al*, 1986). However, most of the naturally occurring mutations in FinP are found in the stem domains, suggesting that these structures are crucial to the inhibitory action of FinP (Finnegan and Willetts, 1971).

Thus far, this discussion has focused on the *finP* gene of the F (Finlay *et al*, 1986) and R1 (Koraimann *et al*, 1991) plasmids only. However, *finP* from the plasmid R100 has also been well characterized, especially at the transcriptional level (Fee and Dempsey, 1986; Dempsey, 1987). R100 differs significantly from F and R1; it expresses two species of FinP, 105 and 180 bases in length (Dempsey, 1987). However, nuclease protection experiments reveal that a 74 base FinP molecule also exists in R100. The 105 base transcript is thought to be analogous to the FinP RNA expressed by F, while the 180 base transcript is unique to R100 (Dempsey, 1987).

III. *finO* - A Protein or an RNA?

The *finO* gene is located at the distal end of the *tra* operon and, as mentioned earlier, is absolutely required for the inhibition of *traJ* expression. Intact *finO* genes can be found in most IncF plasmids, with F being a notable exception. F is *finO*$^-$ by virtue of an IS3 element which interrupts the putative *finO* open reading frame (Yoshioka *et al*, 1987). This naturally derepressed state allows F to transfer at a frequency 1000-fold greater than repressed IncF plasmids, a feature which facilitated its early discovery (Lederberg and Tatum, 1946). The ability of *finO* from other IncF plasmids to complement this deficiency in F indicates that *finO* is not plasmid specific.

Willetts and Maule (1986) identified two alleles of *finO* based on their differential abilities to repress the transfer of F. Group I alleles (R100, R6-5) were found to repress transfer by a factor of 100-1000 fold. Comparatively, group II alleles (R386, ColB2) inhibited transfer by only 20-50 fold. Several group I alleles of *finO* have been cloned and sequenced (Dempsey and McIntire, 1983; Yoshioka *et al*, 1987, 1990; Cram *et al*, 1991) but, prior to the work presented in this thesis, group II alleles have not been characterized, making a direct comparison of the gene products impossible.

An analysis of the published sequences of the *finO* loci reveals an open reading frame which could give rise to a 21.2 kDa protein. In fact, when placed under the control of the *tac* promoter, such a protein product is readily detectable (McIntire and Dempsey, 1987). This open reading frame is in the same orientation as, but downstream of, the *tra* operon and is probably not under the control of the pY promoter. However, McIntire and Dempsey (1987) have characterized a number of internal deletion mutants which argue against the proposal that *finO* encodes a protein. Although these mutants encode a protein product of only 63 to 75 amino acids, as compared to the 168 amino acids encoded by the full length ORF, they still retain *finO* activity. The authors propose that the *finO* gene product is made from the bottom strand, in the opposite orientation from the *tra* operon. It was suggested that the gene product is an untranslated RNA which, based on a short region of homology with *traJ* mRNA (10 bases), interacts with *traJ* mRNA in the same manner as FinP (McIntire and Dempsey, 1987). This thesis will demonstrate that the product of the *finO* gene is indeed a 21.2 kDa protein involved in mediating FinP activity.

Although the mode of action of the *finO* gene product is unknown, several key observations have been made. Two groups (McIntire and Dempsey, 1987; Lee *et al*, 1992) have shown that the intracellular concentration of FinP is increased when *finO* is co-expressed in the cell, despite the observation that *finO* does not affect transcription from the *finP* promoter. Lee *et al* (1992) have extended these observations by demonstrating that *finO* extends the chemical half-life of FinP from less than 3 minutes to greater than 40 minutes. Northern blot analysis of a *lacZ-finP* fusion expressed in a temperature sensitive RNase E mutant strain revealed that the stability of the transcript may be increased at the non-permissive temperature. Based on these observations, the authors suggest that the *finO* gene product may physically protect FinP from cleavage by ribonuclease E (Lee *et al*, 1992).

Prior to the work presented in this thesis, there were no data which convincingly identified the *finO* gene product as either an untranslated RNA or a protein. As a result, the nature of the interaction between the three components of the regulatory system, TraJ, FinP and FinO, was also unknown.

C. Antisense RNA Systems

Most known naturally occurring antisense RNA regulatory systems have been found in prokaryotes, although an increasing number of examples have been identified in eukaryotic systems (Simons, 1993). In prokaryotes, these regulatory systems are associated primarily with extra-chromosomal and mobile elements such as transposons, bacteriophages and plasmids. A number of antisense RNA systems have been very well characterized and are the subject of several recent reviews (Simons, 1993; Nordström *et al*, 1993). Some of the techniques used to study naturally occurring antisense RNA systems are now being used to evaluate the efficiency of artificially designed antisense molecules used for the inhibition of processes such as HIV replication (Homann *et al*, 1993). Three of the best characterized antisense RNA regulatory systems are involved in ColE1 plasmid replication, IS10 transposition, and R1 plasmid replication.

I. Plasmid ColE1 Replication

ColE1 is a bacterial plasmid which replicates under relaxed control, meaning that it is present in multicopy pools of 10-30 copies per chromosome (Conrad and Campbell, 1979). Replication of the plasmid is initiated from an RNA primer (RNA II) which is encoded immediately upstream of the origin of replication (Tomizawa and Itoh, 1981).

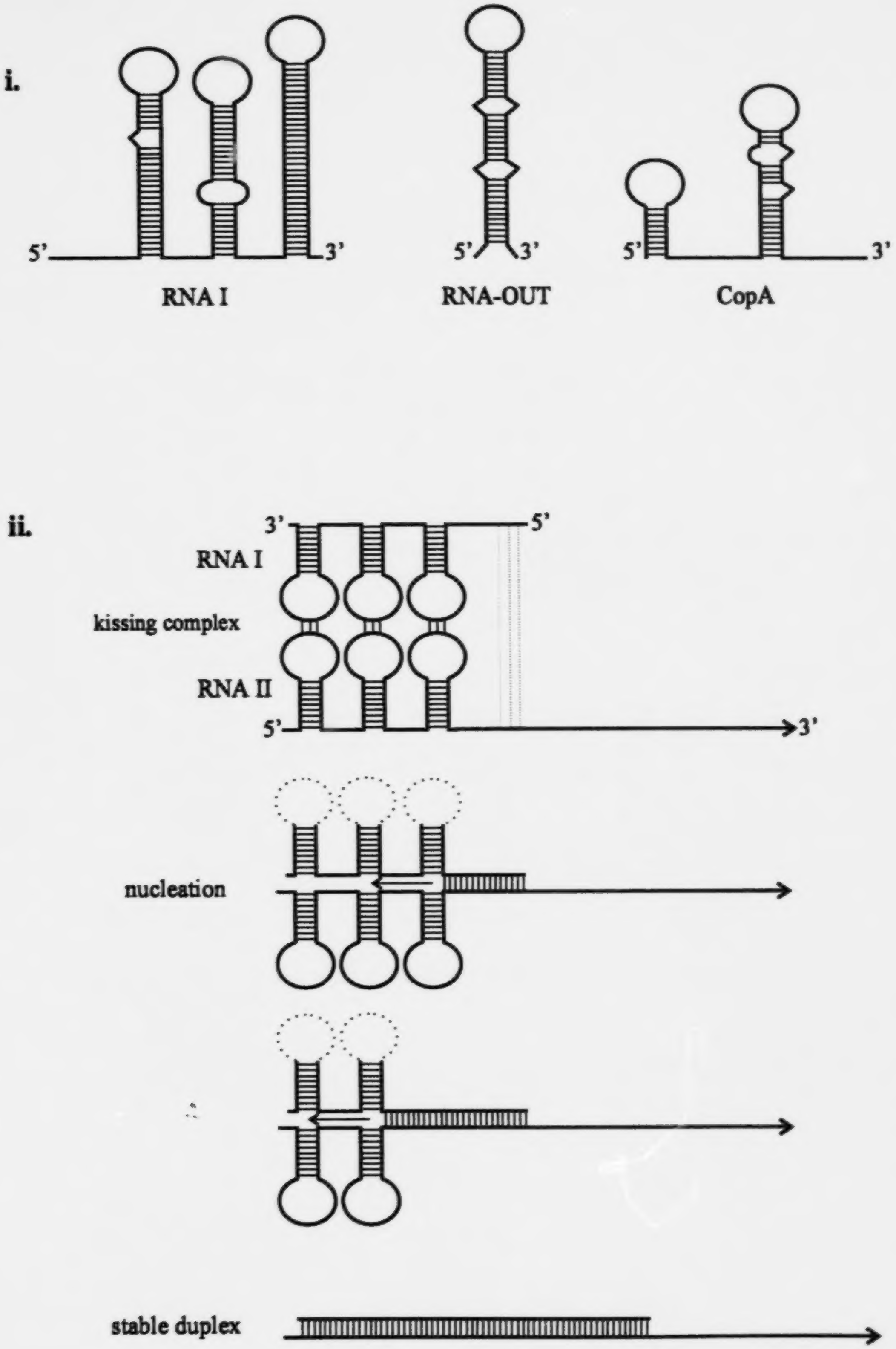
Transcription of RNA II is initiated 555 bp upstream of the origin and yields a transcript which hybridizes with the plasmid DNA. RNA II is subsequently cleaved by RNase H to yield a 555 base primer for DNA synthesis by DNA polymerase I (Itoh and Tomizawa, 1980; Masukata and Tomizawa, 1984).

Conrad and Campbell (1979) demonstrated that there is a second transcript (RNA I) which initiates approximately 450 bp upstream of the origin, proceeds in the opposite direction, and terminates near the initiation site of RNA II transcription. Small insertions made in the coding sequence for this RNA resulted in an increased copy number of the plasmid (Conrad and Campbell, 1979), suggesting a regulatory role for this RNA molecule. Tomizawa and Itoh (1981) confirmed this observation by demonstrating that purified RNA I was able to inhibit cleavage of the RNA II transcript by RNase H, thereby preventing formation of the primer. At the same time, Lacatena and Cesareni (1981) showed that the inhibition of primer formation was the likely result of base pairing between RNA I and RNA II, this being the first identification of an antisense RNA system. This interaction caused a conformational change in RNA II, resulting in the abrogation of its ability to hybridize with the plasmid DNA (Masukata and Tomizawa, 1986).

Extensive structural and functional studies of the RNA I-RNA II pair have revealed a detailed mechanism of interaction between the two RNAs (Tamm and Polisky, 1983; Tomizawa, 1984; Tomizawa and Som, 1984). Figure 1e.i shows that RNA I exists as a tightly folded molecule containing three stem-loop domains and a free 5'-terminal single-stranded region (Tamm and Polisky, 1983). The complementary region of RNA II adopts a similar conformation (Wong and Polisky, 1985). Tomizawa and Itoh (1981) postulated that the RNA I-RNA II interaction occurs via the complementary loop structures of the two RNAs, a hypothesis which was later confirmed (Masakuta and Tomizawa, 1986).

Figure 1e.i: The secondary structures of RNA I, RNA-OUT and CopA. A graphic representation of the RNA structures is shown: bulges and mismatches are shown as triangles (for a single mismatch) or loops (for 2 or more mismatched bases). Regions of base pairing between complementary sequences are shown.

Figure 1e.ii: The step-wise pairing model of RNA I/ RNA II duplex formation. Tomizawa's model of duplex formation is described in detail in the text. The loops of RNA I are shown as dotted lines to demonstrate a continuing interaction with the loops of RNA II.


i.
5'
3'
RNA I
5'
3'
RNA-OUT
5'
3'
CopA
ii.
3'
5'
RNA I
kissing complex
RNA II
5'
3'
nucleation
stable duplex

Tomizawa (1984) studied the kinetics of the formation of a complex between RNA I and RNA II. The formation of the duplex appeared to be a second order reaction, dependent on the concentration of the two component RNA molecules. The rate of duplex formation was easily monitored using polyacrylamide gel electrophoresis, a technique which allowed separation of the duplex from either of the two free RNA species (Tomizawa, 1984). The apparent second order rate constant (K_{app}) of duplex formation was calculated to be $7.1 \times 10^5\ M^{-1}s^{-1}$. This same study revealed that the removal of 5 nucleotides from the 5'-end of RNA I resulted in a drastic reduction in its ability to form a duplex with RNA II, suggesting that the 5'-terminal single-stranded region plays an important role in duplex formation (Tomizawa, 1984).

Based on these data, Tomizawa (1984; 1990a) presents a stepwise pairing model for the binding of RNA I to RNA II (Fig. 1e.ii). Initial recognition and binding occurs between one or more of the complementary loop structures, forming the reversible "kissing complex" (C^*) (Tomizawa, 1985). The kissing complex is quickly converted to the more stable "deep kiss" (C^{**}), an intermediate in which as many as 7 base pairs are formed between the loops of the molecules (Eguchi and Tomizawa, 1990; Tomizawa, 1990a; Eguchi and Tomizawa, 1991). This interaction allows the single-stranded 5'-end of RNA I to interact with its complementary region in RNA II where pairing begins. As the molecules "zip" together, the individual loop-loop contacts are broken and the final stable complex (C^s) consists of an RNA I-RNA II duplex:

$$\text{RNA I} + \text{RNA II} \Leftrightarrow C^* \Leftrightarrow C^{**} \Rightarrow C^s$$

The ultimate result of this interaction is a significant conformational change in the secondary structure of RNA II, such that it is no longer able to hybridize to the plasmid DNA and prime DNA synthesis (Masukata and Tomizawa, 1986).

Recently, Lin-Chao and Cohen (1991) showed that the rate of degradation of RNA I is a key element in the control of ColE1 replication. These authors demonstrated that RNA I is cleaved by RNase E at a position 5 bases from the 5'-end. Interestingly, this processing yields an unstable product (pRNAI$_{-5}$) which is similar to the construct reported by Tomizawa (1984) and is equally impaired in its ability to inhibit primer formation. However, a 5'-triphosphate-terminated homologue of pRNAI$_{-5}$, termed pppRNAI$_{-5}$, was found to repress the priming of DNA synthesis (Lin-Chao and Cohen, 1991), suggesting that RNA I *in vivo* does not require its 5'-terminus as has been proposed by Tomizawa (1984). These authors fail to reconcile this *in vivo* observation with Tomizawa's *in vitro* data.

As is the case with the FinOP system presented above, the ColE1 replication mechanism also involves a protein which, in this case, affects the interaction between RNA I and RNA II (Tomizawa and Som, 1984). This 63 amino acid protein, Rom (for RNA one inhibition modulator), is encoded immediately downstream of the replication origin and has been shown to enhance the inhibitory effect of RNA I on primer formation (Twigg and Sherratt, 1980).

Tomizawa and Som (1984) demonstrated that Rom did not affect the level of transcription of RNA I, but rather that Rom increased the rate of duplex formation between RNA I and RNA II. Interestingly, Rom does not interact with either of the two RNA molecules alone. The protein binds as a single dimer (Tomizawa, 1990b; Eguchi and Tomizawa, 1990) to an early intermediate and inhibits its dissociation, thereby driving the reaction to right:

$$\text{RNA I} + \text{RNA II} + \text{Rom} \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} C + \text{Rom} \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} C^m \overset{k_3}{\Rightarrow} C^s$$

where C represents free kissing intermediates and C^m is C bound by a single Rom dimer. By stabilizing the kissing intermediates, Rom effectively reduces the value of k_{-1}, thus favoring the formation of the duplex. The value of k_{-2} is quite large, suggesting that C and C^m rapidly interconvert. Note that the duplex is extremely stable, therefore the value of k_{-3} is insignificant (Tomizawa, 1990b; Eguchi and Tomizawa, 1990).

The effect of Rom is not overwhelming. Mutations in the Rom coding sequence cause a two-fold increase in the plasmid copy number. Similarly, the inclusion of Rom protein in a duplex reaction doubles the K_{app} from 7.1×10^5 $M^{-1}s^{-1}$ to greater than 1×10^6 $M^{-1}s^{-1}$ (Tomizawa and Som, 1984). The rate of duplex formation is particularly important for this system since this complex must be fully formed before transcription of RNA II proceeds beyond 360 bases (Tomizawa, 1986). After this point, the conformation of RNA II is altered in such a way so that it is no longer affected by RNA I, thus normal primer formation occurs.

II. IS10 Transposition

The tetracycline resistance transposon Tn10 is flanked by the genetically mobile IS10 insertion sequences. IS10 encodes the transposase gene product (Tnp) which mediates transposition of the DNA. The *tnp* gene occupies most of the IS10 sequence and is transcribed from the pIN promoter to yield the RNA-IN mRNA. RNA-IN is subsequently translated into the transposase enzyme. Expression of the *tnp* gene is rate-limiting for transposition and is negatively regulated by a ~70 nt antisense RNA called RNA-OUT (Simons and Kleckner, 1983). RNA-OUT is under the control of the pOUT promoter and is transcribed in the opposite orientation from RNA-IN such that there is a 35 bp overlap at the 5'-end of the *tnp* gene. This overlap includes the *tnp* RBS and translation initiation codon.

The structure of RNA-OUT is considerably simpler than that of RNA I (Kittle *et al*, 1989) (Fig. 1e.i). The molecule consists of a single 21 bp stem domain topped by a loosely structured loop domain. The single-stranded 5'-end of RNA-IN is complementary to the top of the loop domain and the 3'-side of the RNA-OUT stem. The apparent second order rate constant of duplex formation (K_{app}) was measured as 3×10^5 $M^{-1}s^{-1}$ (Kittle *et al*, 1989), approximately 2.5-fold lower than the RNA I-RNA II pair.

Mutational analysis reveals that alterations at the 5'-end of RNA-IN or the top of the loop of RNA-OUT disrupt the pairing reaction, whereas mutations in the RNA-OUT stem had no effect (Kittle *et al*, 1989). Also, the addition of nucleotides to the 5'-end of RNA-IN caused a marked reduction in the rate of duplex formation. Based on these observations, the authors propose that the initial interaction occurs between the 5'-end of RNA-IN and the loop domain of RNA-OUT. Nucleation of RNA pairing is thought to begin with three G-C base pairs and then proceed down the length of the 3'-side of the stem. This process would require that the stem structure be disrupted and also, that the 5'-end of RNA-IN pass through the RNA-OUT loop once for every helical turn of the RNA duplex. This model clearly explains the detrimental effect of adding nucleotides to the 5'-end of RNA-IN since these nucleotides would prevent rotation of the 5'-end through the loop. Mutations which serve to close and stabilize the loop structure also inhibit duplex formation, again because this would effectively prevent passage of the RNA-IN 5'-end through the loop (Kittle *et al*, 1989).

Although the stem domain of RNA-OUT does not appear to be directly involved in duplex formation (Kittle *et al*, 1989), it is the primary determinant for the unusually long (> 60 min) *in vivo* half-life of RNA-OUT (Case *et al*, 1989). Mutations which disrupt the double-stranded nature of the stem domain render the molecule susceptible to exoribonucleases, resulting in a significant decrease in stability. Conversely, mutations which remove mismatches from the stem domain, thereby increasing the length of the double stranded region, cause the molecule to become susceptible to cleavage by the double-stranded ribonuclease, RNase III. These results show that both the double-stranded nature and the presence of bulges in the stem are required for RNA stability (Case *et al*, 1989).

As described earlier, the mechanism by which RNA I prevents primer formation from RNA II involves an alteration in the secondary structure of RNA II, such that it is no longer able to hybridize to the plasmid DNA. In an attempt to uncover the mechanism by which RNA-OUT prevents Tnp expression, Case *et al* (1990) demonstrated that the RNA-IN/RNA-OUT duplex is unstable *in vivo*. As a result, one would expect that a decrease in Tnp expression is the direct result of a decrease in the intracellular level of RNA-IN. While the stability of both RNA molecules is reduced, primarily as a result of cleavage by RNase III, this does not appear to be the primary level of inhibition. Indeed, in an accompanying paper, Ma and Simons (1990) use a sensitive toe-print assay to measure the ability of ribosomes to bind RNA-IN. They show that the formation of a duplex with RNA-OUT occludes the RNA-IN RBS and thereby directly prevents translation initiation. The authors propose that this is the primary mechanism of RNA-OUT mediated inhibition of Tnp expression (Ma and Simons, 1990).

III. Plasmid R1 Replication

The replication frequency of plasmid R1, a member of the IncFII incompatibility group, is regulated by the availability of a rate-limiting protein, RepA. This protein activates the initiation of plasmid replication (Masai *et al*, 1983) and it, in turn, is negatively regulated at both the transcriptional and translational levels. This discussion will focus on the main copy number control system, the inhibition of RepA translation by the antisense RNA, CopA. The *copA* gene is transcribed in the opposite orientation from *repA*, resulting in an RNA which is complementary to the leader region of the *repA* mRNA. This region of complementarity is referred to as CopT (CopA target). Note that

CopT is located more than 80 nt upstream of the *repA* mRNA RBS and translation initiation codon.

The structure of CopA is reminiscent of other well characterized antisense RNAs (Wagner and Nordström, 1986) (Fig. 1e.i). Although CopA lacks a single stranded 5'-tail, stem-loop II is flanked by extended unpaired sequences. The structure of CopT is complementary to that described for CopA (Öhman and Wagner, 1989).

Persson *et al* (1988) determined the second order rate constant of duplex formation (K_{app}) for the CopA-CopT pair to be approximately 1 x 10^6 $M^{-1}s^{-1}$, a value similar to that calculated for RNA I/RNA II. Also, it was shown that the specificity of CopA-CopT interaction lies primarily in loop II of CopA, rather than in loop I or both loops. In the case of RNA I, copy number mutants implicate all three loops in the primary interaction (Tomizawa and Itoh, 1981; Lacatena and Cesareni, 1983).

Duplex formation between CopA and CopT has been shown to occur by way of a stepwise pairing mechanism, similar to that proposed for ColE1 replication (Persson *et al*, 1990a). The initial transient interaction involves only stem-loop II of CopA and nucleation of RNA pairing is thought to begin with the single-stranded spacer located between stem-loops I and II (Persson *et al*, 1990a). Using a series of deletion derivatives of CopA, it was shown that the single-stranded region required for complete duplex formation can be positioned on either side of stem-loop II, provided that the complementary sequence is present in CopT. Also, the base pairing between stem-loop II of CopA and CopT was detected directly using the double-strand specific nuclease, RNase V1. The region involved in formation of the kissing complex is 6-8 nt long and includes bases from both the loop and the top of the stem. The authors postulate that the bulge present near the top of the stem is required to facilitate local melting of the stem, thereby allowing the kissing complex to form (Persson *et al*, 1990a). They note that several antisense RNAs carry similar bulges in the upper part of their major stems. Finally, by performing binding experiments at different salt concentrations and temperatures, Persson *et al* (1990a) show that the binding reaction involves the progressive breaking of intramolecular hydrogen bonds and the formation of intermolecular bonds, as opposed to a complete melting of the RNAs prior to duplex formation.

In an accompanying paper, Persson *et al* (1990b) use a shortened CopA molecule, CopI, which is unable to form a complete duplex with CopT, to dissect the kinetics of CopA-CopT pairing. CopI retains its ability to form the initial kissing complex and, based on this ability, was used to determine the individual rate constants involved in duplex formation:

$$\mathrm{CopA} + \mathrm{CopT} \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} \mathrm{C} \overset{k_2}{\Rightarrow} \mathrm{C^s}$$

Since CopI is completely identical to CopA in stem-loop II, it is reasonable to assume that the kinetics of formation of the kissing complex will be also be similar. Based on the ability to resolve the kissing intermediate on non-denaturing gels, the values of k_1 and k_{-1} were calculated to be ~3.4×10^6 $M^{-1}s^{-1}$ and 1×10^{-5} s^{-1}, respectively (Persson *et al*, 1990b), suggesting that the kissing intermediate is very stable. Since K_{app} was reported to be 3×10^6 $M^{-1}s^{-1}$ in this paper, k_2 is very much larger than k_{-1}, indicating that the rate limiting step in CopA-CopT pairing is the formation of the initial transient complex. Since the duplex forms rapidly once the RNAs have formed the initial transient complex, the efficiency of antisense RNA mediated inhibition of the target is a function of the collision rate of the RNAs and, therefore, a function of the intracellular concentration of the RNAs.

Earlier, it was shown that the RNA-IN/RNA-OUT duplex is a substrate for cleavage by RNase III, although this is not required for the inhibition of Tnp synthesis (Case *et al*, 1990). Similarly, Blomberg *et al* (1990) demonstrated that the CopA-CopT duplex is also cleaved by RNase III both *in vivo* and *in vitro*. This was supported by the observation that the expression of a *repA-lacZ* fusion was increased by 10-fold in an RNase III mutant strain. The authors proposed that cleavage of the duplex by RNase III increases the rate of *repA* mRNA decay, thereby reducing the overall level of RepA synthesis (Blomberg *et al*, 1990). However, in a later communication, Wagner *et al* (1992) showed that a truncated form of CopA, which is able to form a kissing complex, but not a duplex, with CopT, is sufficient for the inhibition of RepA synthesis. Thus, RNase III cleavage of the duplex is not required for R1 plasmid copy number control.

CopT lies in an ORF which has been shown to give rise to a 7 kDa protein product called Tap (translational activator peptide) (Wagner *et al*, 1987; Blomberg *et al*, 1992). The *tap* gene is located immediately upstream of *repA* and both proteins are translated from the same mRNA. The translational stop codon of *tap* overlaps with the *repA* translation initiation codon. A translational start mutation and stop codons inserted in the *tap* gene reduce RepA expression severely. However, Tap is not the active inhibitor of RepA expression since these mutations cannot be complemented in trans by an intact *tap* gene. Specific suppression of the stop codons restores RepA synthesis, indicating that active Tap translation is required for RepA translation initiation (Blomberg *et al*, 1992). The authors went on to demonstrate that *tap* and *repA* are directly coupled at the translational level, such that the ribosomes completing translation of Tap immediately begin translation of RepA. Finally, CopA was shown to directly inhibit Tap translation, presumably by binding to the Tap coding sequence. Blomberg *et al* (1992) present a model of R1 copy number control in which CopA directly inhibits the translation of Tap which, in turn, reduces the level of RepA synthesis as a result of a loss of translational coupling.

IV. Summary

This review of antisense RNA systems has focused on the three most well characterized systems. Note that there are dozens of antisense RNA - target RNA pairs that have been identified and many are being subjected to detailed analysis (Simons, 1993).

The three systems presented here are all similar in some ways and vastly different in others. For example, all of the antisense RNAs exhibit a high degree of secondary structure, a feature which is thought to be crucial to the rapid rate of complex formation (Persson *et al*, 1990). However, the targets of these RNAs vary in their degree of secondary structure, but in every case a single-stranded region has been shown to be important for duplex formation. In RNA I and CopA, these single-stranded regions are located at the 5'-side of the important stem-loop structures whereas, in RNA-OUT, the single-stranded region is provided by the RNA-IN target. The observation that the apparent second order rate constants of duplex formation (K_{app}) are all $\sim 1 \times 10^6$ $M^{-1}s^{-1}$ is not surprising, considering that the rate limiting step in complex formation has been shown to be the formation of the initial transient complex.

The RNA I-RNA II pair is unusual because its binding activity is enhanced by the presence of a third component, the Rom protein. This phenomenon is important for the purposes of this thesis, since the activity of FinP is affected by the presence of the *finO* gene. The interaction between an antisense RNA and a protein has been observed in one other case, the chromosomally derived *micF* antisense RNA (Andersen *et al*, 1989). *micF* is unusual because it is not encoded by the same region of DNA as its target, *ompF* mRNA. *micF* RNA inhibits the expression of OmpF *in vivo*, and *ompF* mRNA and *micF* RNA have been shown to interact *in vitro* (Andersen and Delihas, 1990). Also, gel shifts of *micF* RNA by crude *E. coli* cell extracts reveal that *micF* RNA is specifically bound by an *E. coli* protein. UV-crosslinking analysis has indicated that the size of this protein is approximately 80 kDa. The authors proposed that *micF* RNA inhibits the expression of OmpF as a ribonucleoprotein complex (Andersen and Delihas, 1990).

D. Research Objectives

Extensive genetic analysis of many of the genes of the *tra* operon has yielded a wealth of information concerning their function (reviewed in Frost *et al*, 1994). This is also true for the genes involved in regulation of the *tra* operon, *traJ*, *finP*, and *finO*. These early results clearly showed that both FinP and *finO* are absolutely required for the inhibition of TraJ expression. Lee *et al* (1992) made the crucial observation that *finO*, when expressed in trans with FinP, is able to increase the chemical stability of FinP by more than 15-fold. Also, the co-expression of *finO* and FinP was shown to reduce the steady-state level of *traJ* mRNA by three- to five-fold (Lee *et al*, 1992). However, the mechanism by which these three regulatory molecules interact has remained unexplained.

As described earlier, Willetts and Maule (1986) reported that there appear to be two different alleles of *finO* which inhibit transfer with different efficiencies. In an attempt to uncover the cause of this difference, I cloned and sequenced the *finO* gene from plasmid ColB2 (a representative of the group II alleles). Analysis of the nucleotide sequence of *finO* and also its flanking sequences revealed the major difference between the two allelic groups.

Many sensitive techniques have been described to analyze the nature of the interaction between the components of an antisense RNA system. Using these methods, I studied the structure of the component RNAs, FinP and *traJ* mRNA, and analyzed the kinetics of their interaction. Also, the effect of FinP on *traJ* mRNA *in vivo* was studied using primer extension analysis of the transcripts.

Finally, the FinO protein was purified and used for *in vitro* and *in vivo* analyses to elucidate its function. The complex nature of the interaction between FinO, FinP and *traJ* mRNA was studied and its effects on FinP-*traJ* mRNA duplex formation were examined.

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Chapter 2

Differential levels of fertility inhibition among F-like conjugative plasmids are related to the cellular concentration of *finO* mRNA*

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A. Introduction

Bacterial conjugation is mediated by plasmids which encode all of the functions required for DNA transfer and mobilization. The F factor of *E. coli* is the best studied conjugative plasmid since, in its wild-type state, it is derepressed for transfer (Meynell et al., 1968; Willetts, 1977). ColB2 is a conjugative plasmid belonging to the IncFII incompatibility group, and shares extensive homology with F in the *tra* region (Finlay et al., 1984). Conjugative transfer of F-like plasmids requires the expression of sex pili by the donor cell which initiate close interaction between mating cells (Paranchych and Frost, 1988; Ippen-Ihler, 1989). All of the genes required for pilus formation are encoded by the 33 kb *tra* operon of F-like plasmids.

Transcription of the *tra* operon is positively regulated by the transcriptional activator, TraJ (Willetts, 1977; reviewed in Willetts and Skurray, 1987). TraJ, in turn, is negatively regulated by the FinOP (Fertility Inhibition) system. The *finP* gene of F encodes a 78 nucleotide antisense RNA complementary to the 5' untranslated region of *traJ* (Mullineaux and Willetts, 1985; Dempsey, 1987; Finlay et al., 1986). FinP is thought to inhibit translation of the *traJ* transcript by binding to the mRNA. This inhibitory activity is observed only in the presence of the *finO* gene product, a 22 kDa protein which is encoded at the promoter-distal end of the *tra* operon of other F-like plasmids (McIntire and Dempsey, 1987).

Willetts and Maule (1986) have identified two variants of *finO* with different abilities to repress F transfer. Type 1 *finO* genes exhibit a 100-1000 fold inhibition of F transfer while type 2 *finO* genes inhibit the transfer of F by only 20-50 fold. The nucleotide sequences of the type 1 *finO* genes from plasmids R100 (Yoshioka et al, 1981; 1990) and R6.5 (R. Skurray, personal communication) have been determined and are highly homologous. Also, both plasmids carry identical 0.85 kb open reading frames immediately upstream of the *finO* gene, encoded by *orfC* in R100 and *orf286* in R6.5.

In an attempt to determine the true nature of FinO, we have cloned and sequenced the type 2 *finO* gene from ColB2. Comparison of the nucleotide sequences surrounding the *finO* genes suggests a role for sequences homologous to *orf286*. Simple frameshift and deletion mutants, T7 polymerase expression studies and Northern blotting analysis, reveal a subtle mechanism for regulation of *finO* transcription through base pairing between two genes on the same mRNA message.

B. Results

I. Nucleotide sequence of the ColB2 *finO* gene

The nucleotide sequences of the promoter-distal regions of the conjugative plasmids F, R100 and R6.5 have been determined (Yoshioka et al., 1987; 1990; R. Skurray, personal communication). R100 and R6.5 carry type 1 *finO* genes whereas F is unclassified since it is naturally *finO*⁻ by virtue of an IS3 insertion within the gene. Figure 2a shows a physical map of the R6.5 and ColB2 promoter-distal regions and presents the subclones used in this study.

In an attempt to determine whether the allelic differences observed by Willetts and Maule (1986) were sequence dependent, we have cloned and sequenced the type 2 *finO* gene of ColB2. The sequence of the insert in plasmid pTVB2.1 was determined and the resulting data are presented in Figure 2b together with the derived amino acid sequence.

Analysis of the nucleotide sequence reveals that the ColB2 *finO* gene lies directly downstream of *traX* whereas, in R100 and R6.5, the region between *traX* and *finO* is occupied by *orf286* (or *orfC*) (Yoshioka et al., 1990; R. Skurray, personal communication). It is interesting to note that the sequences surrounding *orf286* in R100 and R6.5 are almost identical to bordering sequences in ColB2, indicating that the gene for *orf286* was either inserted into or deleted from one allele sequence to give the other allele. The extensive homology between type 1 and type 2 *finO* genes suggests that their different phenotypes are not due to sequence dissimilarities. No sequence similarity between either FinO or ORF286 and sequences in the Swissprot database were detected.

II. *orf286* is directly correlated with increased FinO activity

Based on the observation that ColB2 does not carry an *orf286*-like sequence upstream of *finO*, we attempted to determine whether there was a relationship between *orf286* and increased FinO activity.

Figure 2a: Restriction maps of the regions surrounding *finO* in R6.5 and ColB2 and plasmids used in this study.

i. The map of plasmid R6.5

ii. The map of ColB2 in the region of *finO*.

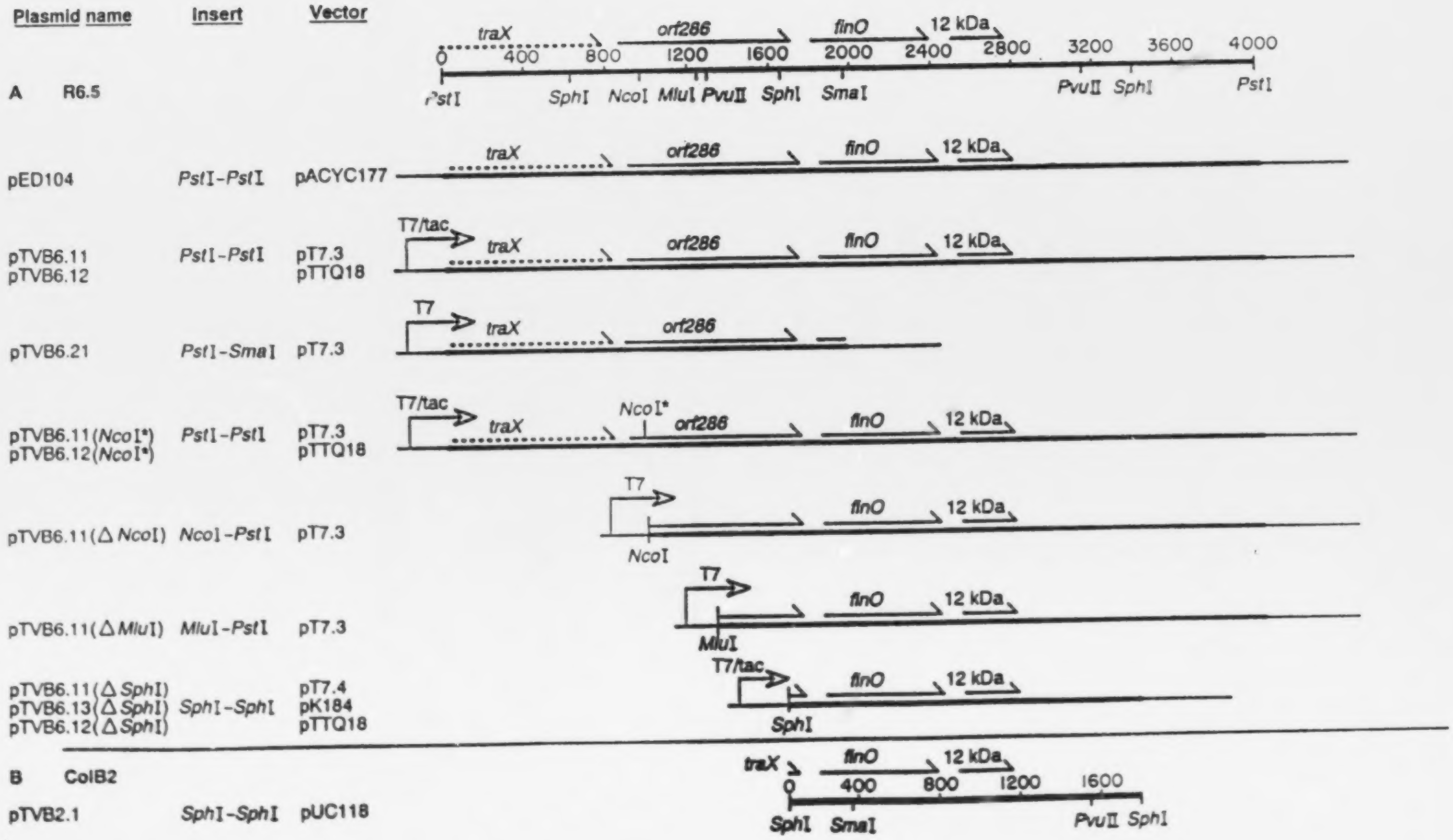
Plasmid name
Insert
Vector
A R6.5
traX
orf286
finO
12 kDa
0
400
800
1200
1600
2000
2400
2800
3200
3600
4000
PstI
SphI
NcoI
MluI
PvuII
SphI
SmaI
PvuII
SphI
PstI
pED104
PstI-PstI
pACYC177
pTVB6.11
pTVB6.12
PstI-PstI
pT7.3
pTTQ18
T7/tac
pTVB6.21
PstI-SmaI
pT7.3
T7
pTVB6.11(NcoI*)
pTVB6.12(NcoI*)
PstI-PstI
pT7.3
pTTQ18
NcoI*
pTVB6.11(Δ NcoI)
NcoI-PstI
pT7.3
NcoI
pTVB6.11(Δ MluI)
MluI-PstI
pT7.3
MluI
pTVB6.11(Δ SphI)
pTVB6.13(Δ SphI)
pTVB6.12(Δ SphI)
SphI-SphI
pT7.4
pK184
pTTQ18
SphI
B ColB2
pTVB2.1
SphI-SphI
pUC118
traX
finO
12 kDa
0
400
800
1200
1600
SphI
SmaI
PvuII
SphI

Figure 2b: Nucleotide sequence of the ColB2 *finO* region. Translation of the *finO* gene is indicated below the sequence. The proposed ribosome binding site (RBS) is underlined (thick) and the *traX* stop codon is underlined (thin). The arrow indicates the approximate site of *orf286* insertion for type 1 *finO* genes. Numbers on the right-hand side indicate the positions of both nucleotides and amino acids. These sequence data appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number X62481.

```
TGACGGTGCTGACCGTTGGCCTGGTGTCCTGTGCAGGGAAATCATTACCCCGTTTCTGGCCCGGGGATTTTTTCTATCTTTGACCGTAAAGGATGATTCGTCAGAGGCAGAACACAGCCC 120
                                                                                                      ↓
GACTTTCTACGCCTGCCATCTGGCTGTGCTGGGCGTTCTGGCACTGTAAAGGGTGTGGTATCTTTGACCGTAAAGGATGATTCGTCAGAGGCAGAACACAGCATGACAGAGCAGAAACGA 240
                                                                                                      METThrGluGlnLysArg 6

CCGGTACTGACACTGAAGCGGAAAACAGAAGGAGAGACGCCTGTCCGCAGCCGGAAAACCATCATCAATGTCACCACGCCACCAAAGTGGAAGGTGAAAAAGCAGAAACTGGCCGAGAAA 360
ProValLeuThrLeuLysArgLysThrGluGlyGluThrProValArgSerArgLysThrIleIleAsnValThrThrProProLysTrpLysValLysLysGlnLysLeuAlaGluLys 46

GCCGCCCGGGAAGCAGAGCTGGCGGCAAAAAAAGCGCAGGCCAGACAGGCGCTGTCCATTTATCTGAACCTGCCCTCACTGGATGAGGCCGTGAATACCCTGAAGCCCTGGTGGCCGGGA 480
AlaAlaArgGluAlaGluLeuAlaAlaLysLysAlaGlnAlaArgGlnAlaLeuSerIleTyrLeuAsnLeuProSerLeuAspGluAlaValAsnThrLeuLysProTrpTrpProGly 86

TTATTTGACGGTGACACGCCCCGGCTTCTGGCCTGCGGTATCCGGGACGTGTTACTGGAAGACGTGGCGCACGGGAATATCCCGCTCTCGCATAAAAAACTGCGCAGGGCGCTGAAGGCC 600
LeuPheAspGlyAspThrProArgLeuLeuAlaCysGlyIleArgAspValLeuLeuGluAspValAlaHisGlyAsnIleProLeuSerHisLysLysLeuArgArgAlaLeuLysAla 126

ATCACCCGTTCAGAAAGCTATCTGTGTGCCATGAAAGCCGGTGCCTGCCGGTATGACACGGAAGGGTATGTGACGGAGCATATTTCTCAGGAGGAGGAAGCGTATGCGGCAGAGCGTCTG 720
IleThrArgSerGluSerTyrLeuCysAlaMETLysAlaGlyAlaCysArgTyrAspThrGluGlyTyrValThrGluHisIleSerGlnGluGluGluAlaTyrAlaAlaGluArgLeu 166

GATAAAATCCGCCGCCAGAACCGGATAAAGGCAGAACTTCAGGCCGTGCTTGATGAGAAATAA 782
AspLysIleArgArgGlnAsnArgIleLysAlaGluLeuGlnAlaValLeuAspGluLys--- 186
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The *Pvu*II-*Sph*1 fragment internal to the *orf286* gene of R6.5 (Figure 2a) was used to probe for the presence of an *orf286*-like sequence in eight conjugative plasmids (data not shown). Table 2a demonstrates a perfect correlation between the presence of a sequence homologous to *orf286* and increased FinO activity, for type 1 alleles of *finO*, and its absence in type 2 alleles as determined by Willetts and Maule (1986).

III. The effect of wild-type *orf286* on FinO expression

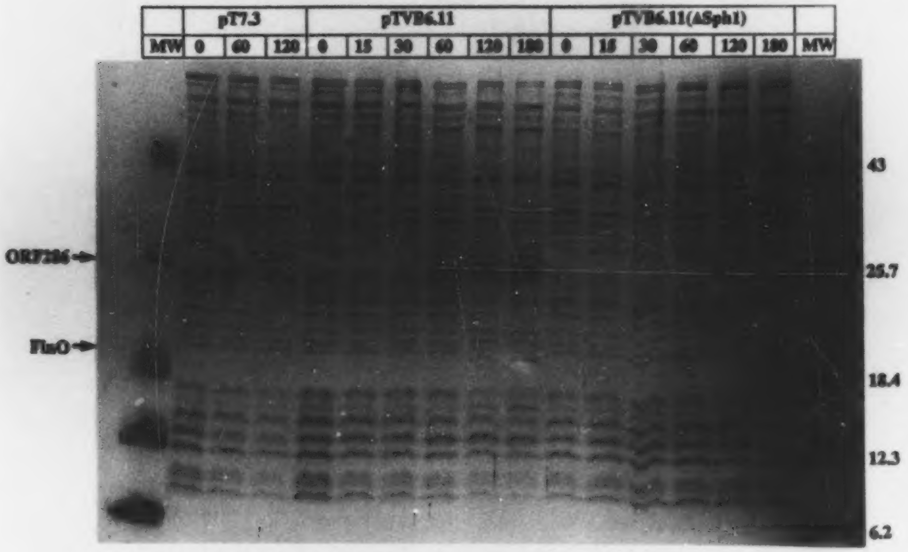
Plasmid pTVB6.11(ΔSph1), a deletion derivative of pTVB6.11 (Figure 2a; *Sph*I deletion), was used to assay the requirement of *orf286* for increased FinO production. Figure 2c shows the products of an induction experiment using plasmids pTVB6.11 and pTVB6.11(ΔSph1), where the sizes of the *orf286* and *finO* gene products correspond to those predicted by the nucleotide sequences (Figure 2c). The 12 kDa protein encoded downstream of *finO* cannot be detected by Coomassie blue staining although it appears as a strong band when labeled with [^{35}S]-methionine. In the presence of *orf286* (plasmid pTVB6.11), a significant amount of FinO was produced towards the end of the 3-hour sampling period. However, in the *orf286* deletion mutant (plasmid pTVB6.11(ΔSph1)), neither FinO nor β-lactamase (the internal control) can be detected. Expression in the presence of [^{35}S]-methionine, followed by SDS-PAGE and autoradiography, reveals that a small amount of both proteins were expressed from pTVB6.11(ΔSph1) and were detectable by ^{35}S-labeling (data not shown). All expression experiments were accompanied by rapid DNA isolations from equivalent samples to demonstrate the presence of the construct in the cells.

Table 2a: The correlation between the presence and absence of *orf286* and the type 1 and type 2 alleles of *finO*.

Plasmid	IncF	Markers	*finO* allele[a]	*orf286*[b]
F*lac*	I	*lac*$^+$	-	-
R386	I	Tc^R	2	-
R1	II	$Ap^RCm^RKm^RSm^RSu^R$	1	+
R6.5	II	$Cm^RKm^RSm^RSu^RHg^RTc^R$	1	+
R100	II	$Cm^RSm^RSu^RHg^RTc^R$	1	+
R136	II	Tc^R	1	+
R538.1	II	$Cm^RSm^RSu^RHg^R$	1	+
ColB2-K77	II	*col*$^+$	2	-
R124 drd	IV	Tc^R	2	-

a. Allele type as defined by Willetts and Maule (1986).
b. Presence (+) or absence (-) of a sequence homologous to *orf286*.
Tc^R, tetracycline resistance; Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Sm^R, streptomycin resistance; Su^R, sulphonamide resistance; Hg^R, hygromycin resistance.

Figure 2c: The effect of *orf286* on FinO expression. Three constructs were examined for protein expression after IPTG induction of the T7 RNA polymerase; pT7.3 the control sample; pTVB6.11, the construct containing both *orf286* and *finO*; and pTVB6.11(ΔSph1), the construct that has the *orf286* gene deleted to the 3' *Sph*I site. See Figure 2a for details. Samples were taken at the indicated time points (in minutes) after IPTG induction. Approximately 0.2 mg of total cellular protein was electrophoresed on a 15% PAG. Gels were stained with Coomassie brilliant blue R stain. Size markers (in kDa) are shown on both sides of the gel with the sizes indicated at the right.


pT7.3
pTVB6.11
pTVB6.11(ΔSph1)
MW
0
60
120
0
15
30
60
120
180
0
15
30
60
120
180
MW
ORF286
FinO
43
25.7
18.4
12.3
6.2

Attempts to increase FinO expression by providing *orf286 in trans* were unsuccessful (data not shown). Cells containing both pTVB6.21 and pTVB6.13(ΔSph1) which contain *orf286* and *finO* respectively under the control of T7 promoters on compatible plasmids, were expressed in *E. coli* BL21(DE3). Although large amounts of ORF286 were produced, FinO could be detected only by labeling with [^{35}S]-methionine, suggesting that the positive effect of *orf286* on FinO production is exclusively a *cis* effect.

IV. The effect of *orf286* on the inhibition of F transfer

Based on these observations, the transfer ability of F-like plasmids could be a function of FinO concentration, which appears to be directly increased by the presence of *orf286 in cis*. In an attempt to determine whether or not *orf286* was responsible for the type 1 FinO phenotype of plasmids R100 and R6.5, we compared the abilities of pTVB2.1, pTVB6.11(ΔSph1), and pTVB6.11 to inhibit the transfer of F using the mating efficiency assay previously described (Frost et al., 1989). Unexpectedly, in all cases, transfer was inhibited by a factor of 500- to 1000-fold as compared to the transfer ability of F in the absence of FinO. This was equivalent to the level of repression that was obtained for the type 1 allele of *finO* of R6.5 carried by the plasmid pED104 (Frost et al., 1989). These results suggested that the sequence differences in the two alleles were not responsible for the differences in repression levels but rather that the concentration of FinO in the cell was important. Supplying the type 2 allele of *finO* from the ColB2 plasmid on a high copy number vector compensated for the low expression of FinO and restored a high level of repression.

V. Construction of *orf286* mutations

The effect of *orf286* on *finO* could occur at either the transcriptional or translational level. In order to assess whether the *orf286* gene product was important in FinO expression, frameshift and deletion mutants of *orf286* were constructed to further characterize the positive effect of *orf286* on FinO production. Plasmid pTVB6.11(Nco1*) was created by cleaving pTVB6.11 with *Nco*I at the unique *Nco*I site, filling in the overhangs and religating (see Figure 2a). A resulting frameshift mutation at the *Nco*I site caused premature termination of the ORF286 protein, in either of the other two reading frames, to give a peptide of predicted molecular weight of 3,000.

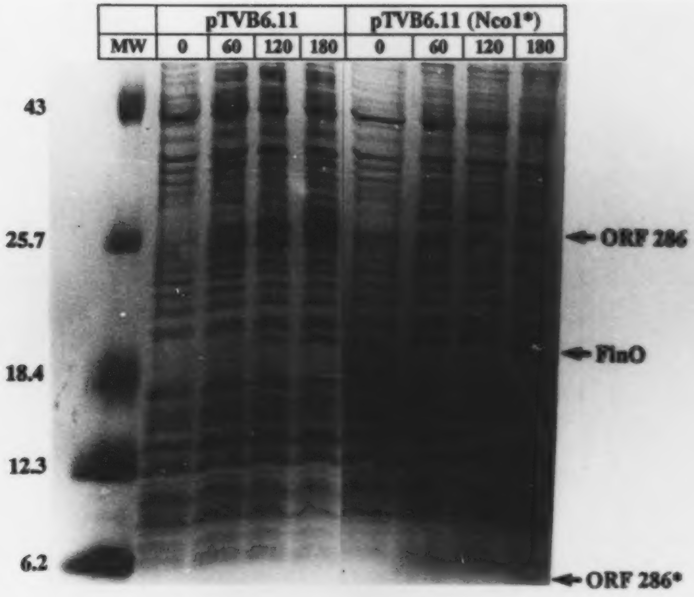
Cleavage of pTVB6.11 by *Eco*R1 and *Nco*1 removed a fragment containing the terminal portion of *traX* and the promoter-proximal portion of *orf286*. The single-stranded overhangs were removed with exonuclease VII to generate blunt ends for religation. The resulting deletion mutant was named pTVB6.11(Δ*Nco*I). A deletion extending to the unique *Mlu*I site of *orf286* was constructed in the same manner and named pTVB6.11(Δ*Mlu*I).

VI. The effect of mutant *orf286* on FinO expression

The frameshift and deletion mutants described above were used to determine whether the positive effect of *orf286* occurred at the translational or transcriptional level. The induction of pTVB6.11(*Nco*1*) (Figure 2d) resulted in the production of an additional induced polypeptide approximately 3 kDa in size which corresponded to the size of the predicted truncated ORF286 gene product generated by the frameshift mutation described above. Despite the absence of a complete ORF286 protein, FinO is produced in large amounts, suggesting that the effect is pre-translational.

Earlier results (Figure 2c) indicated that a deletion to the *Sph*I site (position 780) of *orf286* (plasmid pTVB6.11(ΔSph1)) decreased FinO production drastically. However, deletions up to either the *Nco*I site (position 95) or the *Mlu*1 site (position 365) failed to decrease FinO production (Figure 2d), suggesting that the 415 bp region of *orf286* lying between the *Mlu*I site and the *Sph*I site may be responsible for increased FinO production. Interestingly, all of the mutant *orf286* constructs appeared to produce a significantly larger amount of FinO than the wild-type sequence.

Figure 2d: The effect of frameshift and deletion mutations in *orf286* on FinO expression. A comparison of pTVB6.11 and pTVB6.11(NcoI*) which contains a frameshift mutation in *orf286* by altering the *Nco*I site. Identical results were obtained for the two deletion mutants, pTVB6.11(Δ NcoI) and pTVB6.11(ΔMluI), described in the text. Samples were taken at the indicated time points (in minutes) after IPTG induction. Approximately 0.2 mg of total cellular protein was electrophoresed per lane on a 15% PAG. Gels were stained with Coomassie brilliant blue R stain. Size markers (in kDa) are indicated on the sides of the gels.


pTVB6.11
pTVB6.11 (Nco1*)
MW
0
60
120
180
0
60
120
180
43
25.7
18.4
12.3
6.2
ORF 286
FinO
ORF 286*

VII. The effect of *orf286* on *finO* mRNA concentration

The observation that a translationally inactive *orf286* gene increased FinO production suggested that the *orf286* mRNA was directly responsible for the observed effect. A computer algorithm (RNAFOLD, Zuker and Stiegler, 1981) that is capable of generating secondary structure predictions for 1.2 kb of RNA was used to predict folds in the RNA generated from various constructs (Figure 2e). The results of predictions for the *orf286* and *finO* transcript in tandem suggested that there was extensive base pairing between the two genes and that the RBS for *finO* was exposed in a loop and was accessible to ribosomes. Since *orf286* has no effect *in trans*, the *orf286* mRNA appears to be unable to pair with the *finO* mRNA post-transcriptionally, suggesting that the increased !evel of FinO expression requires the coordinated transcription of both genes in tandem.

An attempt to measure *finO* mRNA stability revealed that the *finO* transcript is present at undetectable levels unless *orf286* is present *in cis* (Figure 2f). Previously, no transcript for *finO* has been found in cells expressing the type 1 allele of R100, in the absence of *orf286* (Dempsey, 1987). This suggested that either the transcript was not efficiently transcribed or it was extremely unstable. Thus, fragments from pTVB6.11 and pTVB6.11(ΔSph1) were transferred to the expression vector pTTQ118 (Stark, 1987) which has a *tac* promoter before the multiple cloning site and the *rrnB* terminator after it, allowing transcription of RNAs of a defined length. In this experiment, clones of *finO* with (pTVB6.12) and without *orf286* (pTVB6.12(ΔSph1)) and pTVB6.12(NcoI*)) were tested for mRNA stability by inducing transcription from the *tac* promoter upon addition of IPTG. The rate of decay of the RNA was followed after the addition of rifampicin, which prevented the initiation of new transcripts. *finO* mRNA was detected using a synthetic oligonucleotide probe described in "Experimental procedures". Pre-induction samples did not contain detectable levels of *finO* mRNA (data not shown), indicating that the only active promoter in the system was the plasmid T7 promoter. The predicted 4.0 kb transcripts from pTVB6.12 and pTVB6.12(Nco1*) were visible only on an overexposed autoradiograph. The two bands of RNA, approximately 1.8 kb and 2.0 kb in size, are likely degradation products of the original 4.0 kb transcripts. Note that the frameshift mutation in *orf286* (plasmid pTVB6.12(*Nco*I*)) does not affect the concentration of the *finO* mRNA. Our inability to detect the predicted 2.0 kb transcript from pTVB6.12(ΔSph1) indicates that this transcript is either not synthesized or is extremely unstable, thereby preventing high-level translation and FinO protein synthesis.

Figure 2e: Computer analysis of the secondary structure in the *orf286/finO* region. This 1190 nucleotide RNA fragment starts 40 bp downstream of the *orf286* start site, and ends in the middle of the *finO* gene. Bases are numbered as in Figure 1. All relevant restriction sites are indicated. The *orf286* stop codon, the *finO* start site, and the *finO* RBS are also marked. The long arrows on either side of the structure indicate the direction of transcription/translation. Double lines indicate the regions of pairing between *finO* and *orf286*. The secondary structure prediction was generated by RNAFOLD (Zuker and Stiegler, 1981) on the University of Wisconsin GCG package (Devereux, 1984) and visualized by *LoopViewer* (Gilbert, 1990).

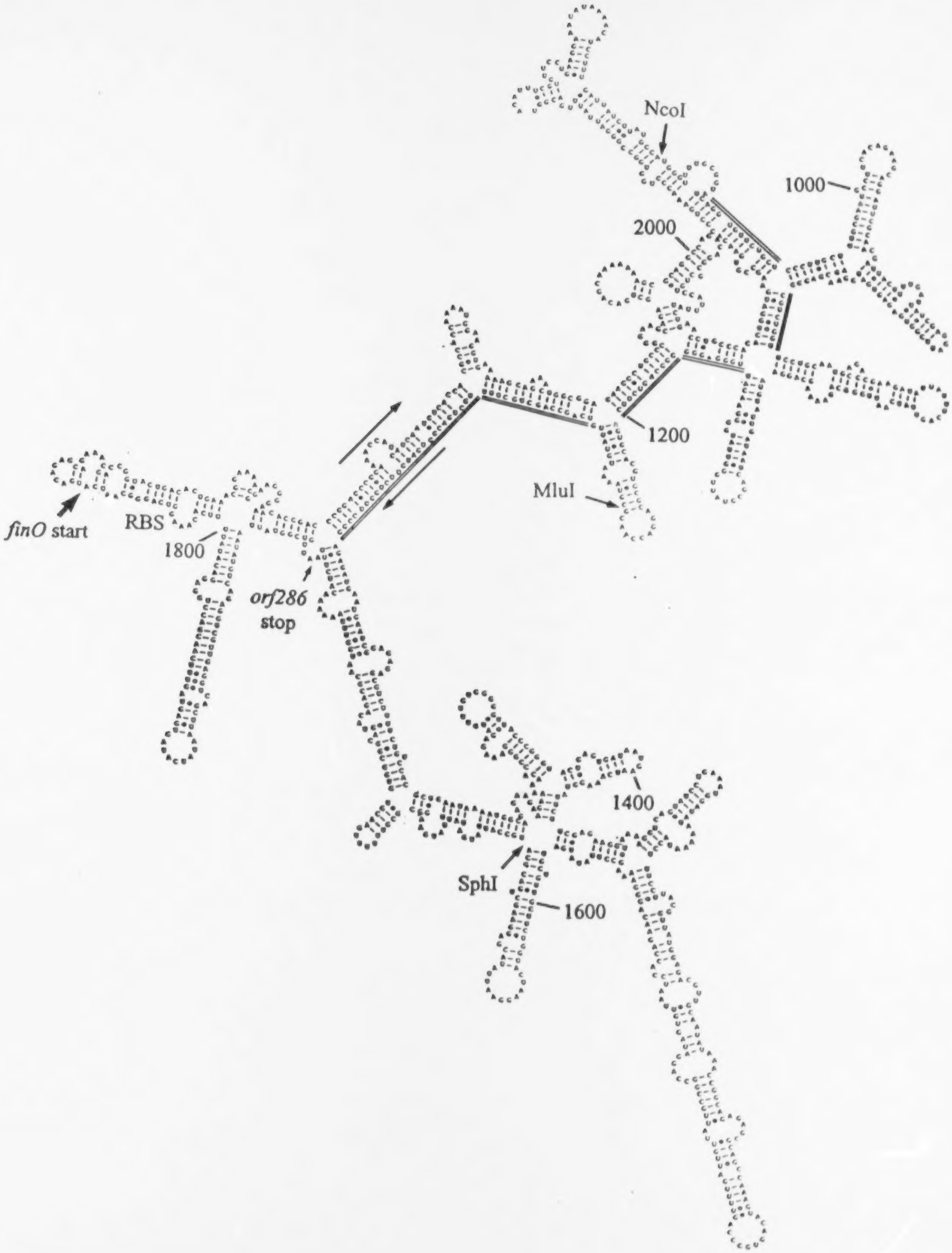
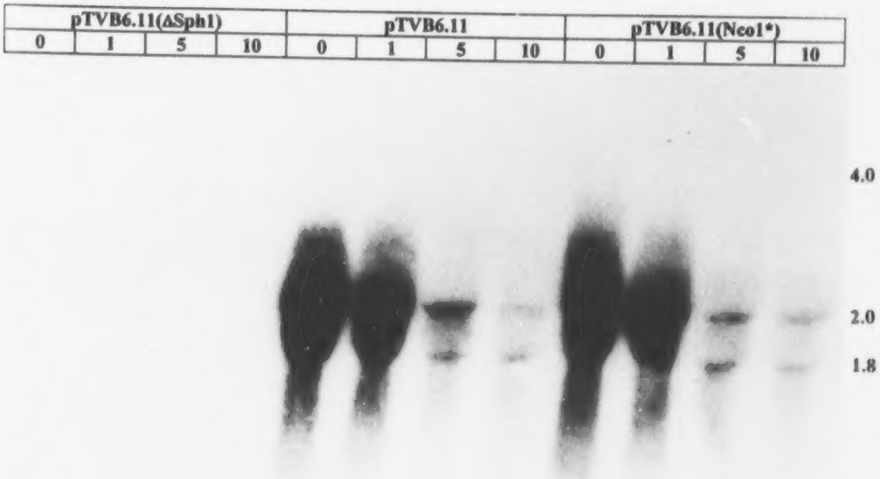

NcoI
1000
2000
1200
MluI
finO start
RBS
1800
orf286
stop
1400
SphI
1600

Figure 2f: Half-life of the *finO* transcript with and without *orf286*. Samples were taken at the indicated time points after rifampicin addition. Approximately 10 μg of total cellular RNA was electrophoresed per lane on a 1% denaturing agarose gel. RNA was transferred onto a Hybond-N nylon membrane and hybridized with the TVB#4 primer, which is complementary to *finO*, as a probe. Samples were taken at the indicated time points (in minutes) after rifampicin addition. Size markers (in kb) are indicated at the right side of the gel.


pTVB6.11(ΔSph1)
pTVB6.11
pTVB6.11(Nco1*)
0
1
5
10
0
1
5
10
0
1
5
10
4.0
2.0
1.8

C. Discussion

We have determined that the differential levels of fertility inhibition among F-like plasmids observed by Willetts and Maule (1986) are not due to sequence differences within the *finO* gene but could be due to an upstream sequence called *orf286* in the R6.5 plasmid. The perfect correlation between the presence of an *orf286*-like sequence and increased FinO activity prompted us to further elucidate the role of this previously uncharacterized gene.

Preliminary protein expression studies indicated that *orf286* was required for enhanced FinO expression. ORF286 protein was very highly expressed both in the presence and absence of *finO*, but when *orf286* was provided *in trans*, its positive effect was not detected, even though a large amount of the ORF286 protein was synthesized. The observation that *orf286* functioned only *in cis* suggested that its effect was at the transcriptional level.

Efforts to abolish the positive effect of *orf286* by generating translationally inactive *orf286* mutants were unsuccessful. Surprisingly, frameshift and deletion mutants of *orf286* were still able to enhance FinO expression, even more so than the wild-type sequence. A 415 bp region between the *Mlu*I site and the *Sph*I site within *orf286* was found to be sufficient to confer the *orf286*$^+$ phenotype. These results suggest that the *orf286* portion of the transcript affects the *finO* portion by encouraging the *finO* mRNA to adopt a conformation that increases its stability as well as access to its RBS. Computer-generated secondary structure predictions for RNA containing *orf286* and *finO* suggested that there was extensive base pairing between the two regions of the transcript and that the RBS for *finO* was exposed in a loop which would provide access to ribosomes, without mandatory translation of the *orf286* gene to open up the secondary structure in the RNA. The increased production of FinO in the presence of mutant *orf286*, as compared to the wild-type, may be due to a lack of disruption of the stabilizing RNA structure by translating ribosomes.

The relationship between mRNA stability and protein expression has recently been demonstrated for the *tra* operon (Koraimann and Hogenauer, 1989). The most highly expressed protein, the pilin subunit (encoded by *traA*), is translated from the most stable portion of the transcript. Our results suggest that the level of FinO present in the cell is also a function of transcript stability. However, Figure 2c demonstrated that the attempted expression of FinO in the absence of *orf286* also reduced the expression of β-lactamase. Thus the *finO* transcript is either very unstable and promotes the degradation of the downstream *bla* transcript, or it contains a transcriptional terminator which is disrupted by the presence of *orf286*.

The correlation between increased cellular FinO concentration and increased levels of fertility inhibition explain why the mating assays, with and without *orf286*, generated identical results. The multicopy vector provided sufficient FinO to repress *traJ*, even in the absence of *orf286*. While the nature of the interaction between the FinOP system and the *traJ* mRNA is unknown, our current model of the FinOP system involves the stabilization of FinP by protecting it from degradation perhaps by RNase E (Lee *et al*, in preparation) even in the absence of the *traJ* transcript.

D. Materials and methods

I. Bacterial strains, plasmids and media

Recombinant plasmids, described below, were transformed into *E. coli* DH5α, JC3272, M176 (JC3272 containing the F plasmid JCFLO) or BL21(DE3) (Studier and Moffatt, 1986). Plasmids pT7.3 and pT7.4 (Tabor and Richardson, 1985) were provided by Stan Tabor (Department of Biological Chemistry, Harvard Medical School, Boston, MA). Plasmid pK184 (Jobling and Holmes, 1990) was provided by Mike Jobling (Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD). Plasmid pTTQ18 (Stark, 1987) was obtained from Amersham (Arlington Heights, IL). Conjugative plasmids F (JCFLO) and ColB2 were as described previously (Achtman et al., 1971; Finlay et al., 1984). All cloning experiments were performed using pUC118 (Vieira and Messing, 1987). Chimeric plasmids used and generated by this study are listed in Figure 2a. LB (Luria-Bertani) broth was as described previously (Maniatis et al., 1982). Antibiotic concentrations were 25 μg ml^{-1} for both ampicillin and kanamycin, and 40 μg ml^{-1} for spectinomycin.

II. Recombinant DNA techniques

All DNA manipulations were performed as described in Sambrook et al. (1989) and Ausubel et al. (1987 with updates). All enzymes were supplied by Boehringer Mannheim (Laval, Que) except Exonuclease VII which was supplied by BRL (Bethesda, MD.).

III. Southern Blotting Analysis

The occurrence of *orf286* among several F-like conjugative plasmids was determined via Southern blotting experiments. The internal *Pvu*II-*Sph*I fragment of *orf286* from pED104 (see Figure 2a) was labeled by random primers and hybridized to Southern blots of the plasmids described in Table 2a. After at least 10 hours hybridization at 60°C in 2.5 X SSC, 5X Denhardt's solution (Sambrook et al., 1989), 1.5 % SDS and 50% formamide, the blots were washed at 50°C in 0.1% SDS, 5 x SSC. Membranes were exposed to Kodak X-AR5 film at room temperature for 2 to 12 hours. Hybridization to a 4 Kb *Pst*I fragment indicated the presence of an *orf286* -like sequence.

IV. DNA sequence analysis

A 2.0 kb *Sph*I fragment containing the ColB2 *finO* gene was cloned into pUC118, named pTVB2.1, and sequenced using the Sequenase kit (United States Biochemical Corporation). Computer-assisted sequence analysis was done using the PC/Gene program of the University of Geneva , Switzerland, the University of Wisconsin Genetics Computer Group (Devereux, 1984), and the *LoopViewer* RNA secondary structure viewing program (Gilbert, 1990).

V. Protein expression

The gene products expressed by the pT7.3 and pT7.4 chimeras were induced as follows. *E. coli* BL21(DE3) transformed with the appropriate chimeras was grown in LB broth to an OD_{600} of 0.3 at 37°C. 10 µl isopropyl -β-D-thiogalactopyranoside (IPTG, 40 mM) was added to 1 ml cultures to initiate induction. Samples were taken at the indicated time points (sample size = 0.2 OD_{600}), centrifuged, lysed with sample buffer, and subjected to SDS-PAGE on a 15% gel. All gels were stained using Coomassie brilliant blue R stain.

VI. Construction of an *in trans* protein expression system

A *Pvu*II-*Eco*RI fragment from pTVB6.11(ΔSph1) containing the entire insert in addition to the T7 promoter, was subcloned into the *Sma*I-*Eco*RI sites of the pK184 multiple cloning site. The resulting construct, pTVB6.13(ΔSph1), was able to express FinO by

IPTG induction from a plasmid carrying a p15a origin of replication and kanamycin resistance. pTVB6.13(ΔSph1) was co-transformed with pTVB6.21 into BL21(DE3) and both plasmids were stably maintained under antibiotic selection. The genes encoded on both plasmids could therefore be induced as described above.

VII. RNA isolation and Northern blotting

Cells were grown to an OD_{600} of 0.75. IPTG (40 mM) was added to a final concentration of 0.5 μM. After five minutes of induction, rifampicin (20 mg ml^{-1} dissolved in methanol; Sigma, St. Louis, MO) was added to a final concentration of 200 μg ml^{-1}. At the indicated time points, 1.5 ml samples were withdrawn, centrifuged, and the pellets quick frozen at -70°C. RNA was isolated by the hot phenol method as previously described (Frost et al., 1989).

RNA was electrophoresed on denaturing agarose gels as previously described (Frost et al., 1989) and transferred from gels to Hybond-N nylon membrane (Amersham) using a semi-dry electro-blotting apparatus (Bio-Rad, Richmond, CA) set at 3 mA/cm^2 gel surface area for 1 hour.

VIII. Oligonucleotide end-labeled probes

The *finO* transcript was detected on Northern blots using a ^{32}P-labeled oligomer which was complementary to a 24 bp region encompassing nucleotides 171-195 downstream of the *finO* translational start site. The primer was labeled with ^{32}P-ATP using T4 polynucleotide kinase according to the method described in Sambrook et al. (1989). Blots were prehybridized for at least two hours at 55°C in 2.5 X SSC, 5X Denhardt's solution (Sambrook et al., 1989), 1.5% SDS, and 100 μg/ml of *E. coli* Strain W tRNA type XX (Sigma). The blots were hybridized for at least 6 hours at 55°C before washing in 1 X SSC, 0.1% SDS at room temperature for 10 minutes. Autoradiography was performed at -70°C in the presence of an intensifying screen, using Kodak X-AR5 film.

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Chapter 3

Structural and functional analyses of the FinP antisense RNA regulatory system of the F conjugative plasmid*

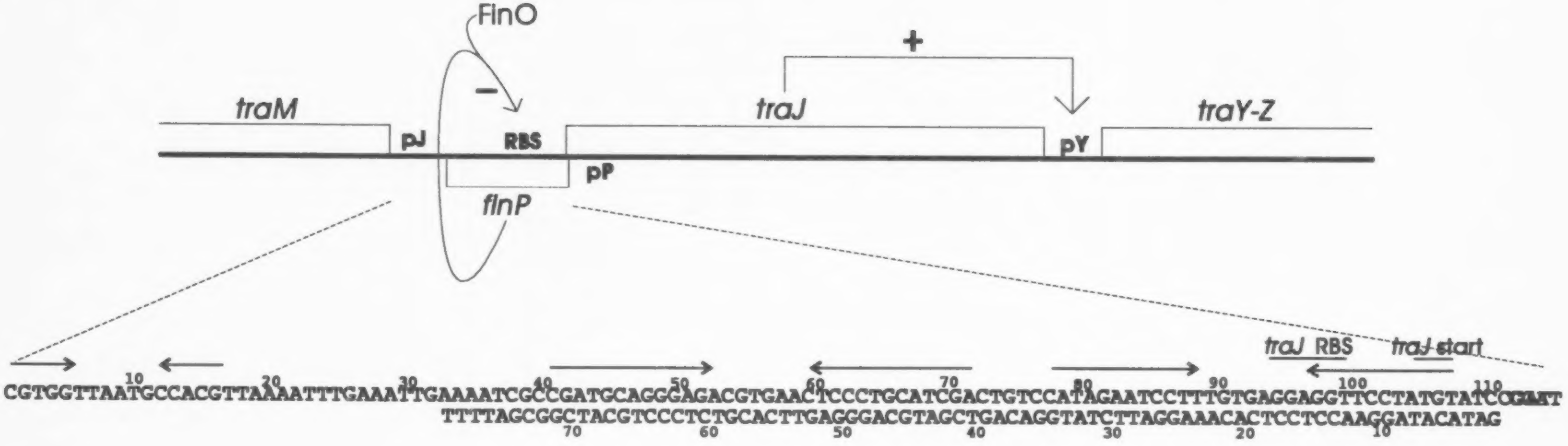
* A version of this chapter has been published: van Biesen, T., Söderbom, F., Wagner, E.G.H., and Frost, L.S. (1993) *Mol. Microbiol.* **10**:35-43.

A. Introduction

Member plasmids of the IncFI incompatibility group require more than 30 genes for the conjugal transfer of DNA. Most of these genes are encoded by a single large (~33 kbp) operon called the *tra* operon. For a review see Ippen-Ihler and Minkley (1989). Transcription of *tra* is activated by the product of the *traJ* gene, which lies immediately upstream of the operon. TraJ synthesis, in turn, is negatively regulated by the fertility inhibition genes, *finO* and *finP* (reviewed by Willetts and Skurray, 1987). The *finP* gene product is encoded from the strand complementary to *traJ* (Fig. 3a), resulting in a 78 base untranslated RNA molecule (FinP) that is fully complementary to part of the 5'-untranslated region of the *traJ* mRNA (Mullineaux and Willetts, 1985; Dempsey, 1987; Finlay *et al.*, 1986). The FinP antisense RNA is thought to inhibit TraJ synthesis by forming a stable duplex with the *traJ* mRNA and thereby occluding its ribosome binding site (RBS). This mechanism of direct translation inhibition has been demonstrated for RNA-OUT antisense RNA of IS10 (Ma and Simons, 1990). In contrast to other known antisense RNA systems, FinP-mediated repression occurs only in the presence of a second gene, *finO* (Finnegan and Willetts, 1973), which encodes a 22 kDa protein (van Biesen and Frost, 1992). Previous results have suggested that FinO increases the chemical stability of FinP (Lee *et al*, 1992) and thus increases the efficiency of fertility inhibition. The nature of the interaction between these two gene products has not yet been described.

Antisense RNA-mediated gene regulation has been implicated in the control of a variety of prokaryotic cellular processes ranging from the control of copy number of accessory elements (for a recent review see Nordström *et al*, 1993) to the regulation of septum formation during cell division (Tetart and Bouche,1992). The mechanisms by which antisense RNAs inhibit gene expression are of great interest since these molecules show promise as potential therapeutic agents (for a review see Neckers *et al*, 1992).

Figure 3a: The control region of the *tra* operon. + and - indicate positive and negative regulatory effects respectively. RBS= ribosome binding site; pP, pJ and pY represent the promoters for the *finP*, *traJ* and *traY* transcriptional units respectively. The sequences of the *traJ* 5'-untranslated region and FinP are shown below. Nucleotides are numbered from the transcription initiation sites. Inverted repeats are shown by arrows over the sequence and are labeled with an alphanumeric code for easy reference.


FinO
+
−
traM
pJ
RBS
traJ
pY
traY-Z
pP
finP
traJ RBS
traJ start
10
20
30
40
50
60
70
80
90
100
110
CGTGGTTAATGCCACGTTAAAATTTGAAATTGAAAATCGCCGATGCAGGGAGACGTGAACTCCCTGCATCGACTGTCCATAGAATCCTTTGTGAGGAGGTTCCTATGTATCCGAAT
TTTTAGCGGCTACGTCCCTCTGCACTTGAGGGACGTAGCTGACAGGTATCTTAGGAAACACTCCTCCAAGGATACATAG
70
60
50
40
30
20
10

The *in vitro* kinetics of RNA duplex formation for several systems have been investigated in detail. Tomizawa (1984) demonstrated that the apparent second order rate constant (k_{app}) of plasmid ColE1 RNA I/RNA II duplex formation is close to 10^6 $M^{-1}s^{-1}$. Persson *et al* (1988; 1990a,b) and Kittle *et al* (1989) have reported similar k_{app} values for the CopA/CopT and RNA-IN/RNA-OUT systems respectively. Persson *et al* (1990a,b) have extended their analyses by defining a number of intermediates that exist during the transition from two free RNA species to the final stable duplex. The formation of a stable intermediate termed the "kissing complex" was shown to be the rate limiting step during duplex formation. In the case of CopA/CopT, complete duplex formation was not required for inhibition of *repA* repression (Wagner *et al*, 1992). The kissing complex is a result of the interaction between the loops of the two RNA species, which could explain the phenotypic effects of mutations in the corresponding loop regions of the FinP/TraJ system (Koraimann *et al*, 1991; Finlay *et al*, 1986). The final step in the pairing pathway, the transition from the kissing complex to a full RNA duplex, is essentially irreversible.

The observation that FinP affects TraJ expression only in the presence of *finO* suggests the possibility that the FinO protein is directly involved in duplex formation. Only one other example of protein involvement in antisense RNA function has been described: the Rom protein of plasmid ColE1 has been shown to stabilize the RNA I/RNA II kissing complex, thereby further reducing the plasmid copy number (Tomizawa and Som, 1984; Eguchi and Tomizawa, 1990; Tomizawa, 1990). However, Rom has been found to interact only with the kissing complex and not with either of the free RNAs, while the *finO* gene product has a specific effect only on FinP (Lee *et al*, 1992). Also, Rom is dispensable for RNA I function while FinO is absolutely required for FinP function.

Previous work on FinP, from both plasmids R1 (Koraimann *et al*, 1991) and F (Frost *et al*, 1989), has identified a series of mutations that appear to correlate well with the computer- predicted structure of the molecule. In addition, phylogenetic comparisons of five alleles of FinP (Finlay *et al*, 1986) reveal that specificity for *traJ* mRNA binding is conferred by the proposed loop regions, suggesting that the remaining sequences involved in stem formation are conserved in order to maintain the secondary structure. These results support the observation that the initial recognition of the target RNA occurs through one or more loops of the antisense RNA (Tomizawa, 1984; Persson *et al*, 1990a,b).

Here we present the preliminary biochemical characterization of the FinP - TraJ antisense RNA system. Using transcripts generated *in vitro* and structure-specific ribonucleases, we have elucidated the secondary structures of both FinP and its target, the 5'-untranslated region of *traJ* mRNA. Both molecules contain the characteristic stem-and-loop structures seen in comparable systems. In addition, we have measured the *in vitro* kinetics of duplex formation in the absence of FinO. The observed k_{app} of 5×10^5 $M^{-1}s^{-1}$ is similar to previously characterized RNA pairs suggesting that FinO is not required for RNA duplex formation

B. Results

I. Secondary structure analysis of the FinP antisense RNA

Computer analysis and phylogenetic comparison of the sequences of the *finP* gene have been used to predict a structure for the FinP RNA *in vivo* (Koraimann *et al*, 1991; Frost *et al*, 1989; Finlay *et al*, 1986). We have analyzed the secondary structure of FinP *in vitro* using structure specific endoribonucleases and we report here that the suggested structure correlates closely with those proposed previously. FinP RNA was synthesized *in vitro* and labeled at its 5'-terminus with γ-^{32}P-ATP. The gel-purified transcript was subjected to partial digestion (<1 cleavage per molecule) by endoribonucleases A, T1, T2, and V1 (Fig. 3b.i).

RNase T1 cleaves preferentially at guanosines in single-stranded regions of the RNA. Since there are no unpaired G residues in stem-loop 1, the first such cleavage is seen at G_{35}, which is the first position in the 4-base spacer located between stems I and II (Fig. 3b.ii). Weaker cleavages appear on the 5'-side of stem II (positions 39, 42, 45, 48-50) and increase in intensity in loop II (positions 52, 55, 58, and 59). We are unable to explain the cleavage of the paired Gs in stem II and the unpaired Cs and Us in loop II (C_{55}, C_{57}, U_{59}). G_{39} and G_{52} are strongly cleaved perhaps as a result of "breathing" of the duplex at the ends of the stem. Cleavage of the single G in the 3'-tail of FinP (G_{74}) was also detected.

The preferential cleavage by RNase T2 of all unpaired bases serves to accurately identify single-stranded regions of RNA. Loop I is easily identified due to strong cleavages at positions C_{16} to A_{20}. Unexpectedly, positions between C_{21} and A_{24} were not cleaved by any of the nucleases used in this analysis (Fig. 3b.i). Digestion with RNase A, which primarily cleaves at unpaired pyrimidines, reveals that C_{21} is indeed unpaired (data not shown; sites labeled in Fig. 3b.i).

Figure 3b: Secondary structure analysis of FinP RNA.

i. Partial digests of FinP RNA with nucleases T1, T2, and V1. The number of units of enzymes used in each digestion is indicated above each lane. The main structural features of the molecule are shown to the left and right. The size standard is an alkaline hydrolysis ladder of FinP (see Experimental Procedures). Stems and loops are labeled with the alphanumeric code shown in Figure 1. Samples were electrophoresed in duplicate: the set of lanes on the left-hand side were loaded 1 hour prior to those on the right to enhance resolution of the larger cleavage fragments.

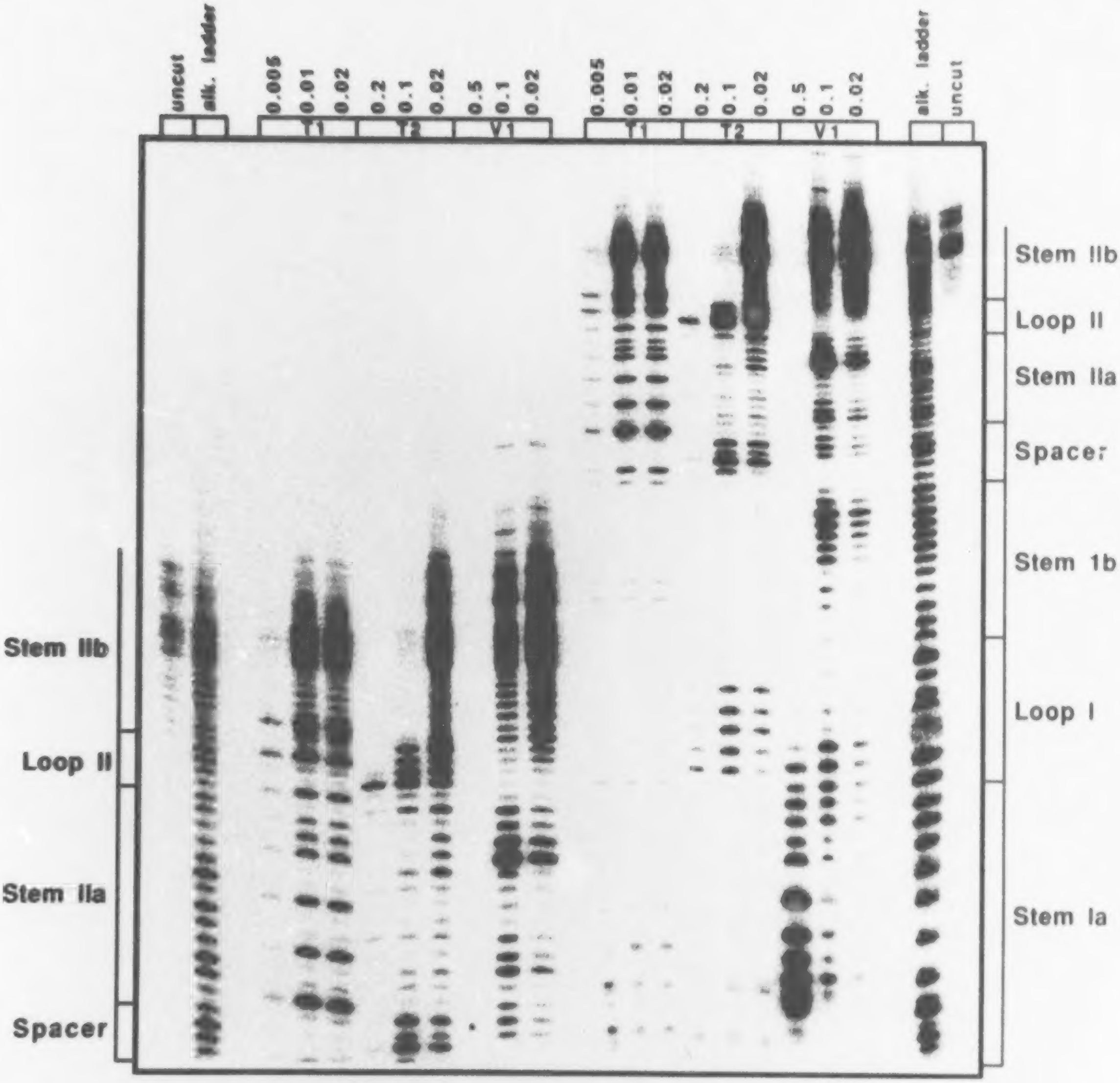
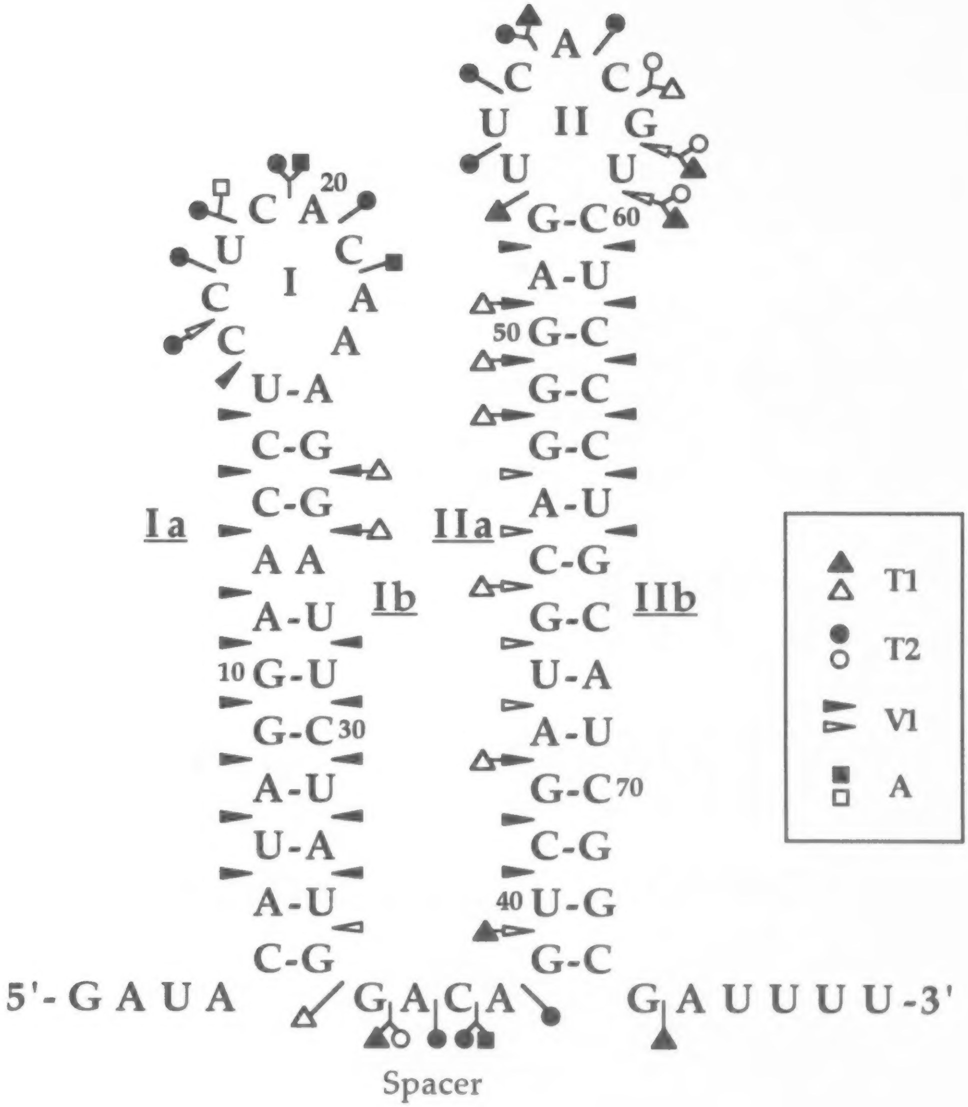

uncut
alk. ladder
0.005
0.01
0.02
0.2
0.1
0.02
0.5
0.1
0.02
T1
T2
V1
0.005
0.01
0.02
0.2
0.1
0.02
0.5
0.1
0.02
T1
T2
V1
alk. ladder
uncut
Stem IIb
Loop II
Stem IIa
Spacer
Stem IIb
Loop II
Stem IIa
Spacer
Stem 1b
Loop I
Stem Ia

Figure 3b: ii. Secondary structure of FinP RNA. The graphical representation shown here is based on previously described computer predictions, phylogenetic analysis (see text) and the nuclease digests shown in (i) and other similar experiments. The cleavage positions are represented by symbols (see inset): open symbols indicate weak cleavages and solid symbols indicate strong cleavages. Nucleotide positions are numbered as in Figure 1.



I
II
Ia
Ib
IIa
IIb
10
20
30
40
50
60
70
5'-G A U A
G A C A
G A U U U U-3'
Spacer
T1
T2
V1
A

All of the bases in the spacer (G_{35} to A_{38}) and in loop II (U_{53} to U_{59}) are efficiently cleaved by RNase T2, while both stems I and II remain undigested (note that weak cleavages by RNase T2 occur in stem II only at high enzyme concentrations).

The double-strand specific RNase V1 was used to identify stems I and II of the FinP molecule. Cleavage was observed at virtually every position of stem I with one notable exception: position A_{27} of the A_{12}-A_{27} "mismatch" was resistant to V1 digestion (Fig. 3b.i), suggesting that these unpaired residues disrupt the RNA helix. Cleavage of stem II was less uniform in that most of the positions on the 5'-side of the stem were cleaved while the 3'-side was only partially digested. This phenomenon of asymmetric digestion was also observed with stem 1 of CopA (Wagner and Nordström, 1986) and, more recently, for RNA I of IncB plasmids (Siemering *et al*, 1993).

II. Secondary structure analysis of the 5'-untranslated region of *traJ* mRNA

Since the FinP target on TraJ RNA is located proximal to the 5'-end of the molecule, it was possible to analyze the structure of this region using structure-specific endoribonucleases (Fig. 3c). However, the sensitivity of the analysis is decreased slightly as a result of the 32-nucleotide sequence preceding the FinP target. We were interested in determining whether the target also carries the double stem-loop structure and whether it is affected by long range RNA-RNA interactions with a downstream sequence in the first 211 nucleotides of the *traJ* RNA. Truncated runoff TraJ transcripts (211 nt in length) were transcribed *in vitro* by T7 RNA polymerase and subjected to digestion (<1 cleavage per molecule) by RNases T1, T2, and V1 (Fig. 3c.i).

RNase T1 strongly cleaves at G_{26} and G_{32} in the region preceding the FinP binding site. Guanosines at positions 3, 5, 6 and 12 are not cleaved since they form a small 6 bp V1-sensitive stem at the start of the transcript (Fig. 3c.ii). At the DNA level, this inverted repeat has high homology to the binding site for the cyclic AMP receptor protein cAMP-CRP (Paranchych *et al*, 1986).

Figure 3c: Secondary structure analysis of TraJ211 RNA.

i. Partial digests of TraJ211 RNA with nucleases T1, T2, and V1. The number of units of enzyme used in each digestion is indicated above each lane. The main structural features of the molecule are shown to the right. The size standard is an alkaline hydrolysis ladder of TraJ211 RNA (see Experimental Procedures). Stems and loops are labeled with the alphanumeric code shown in Figure 1.

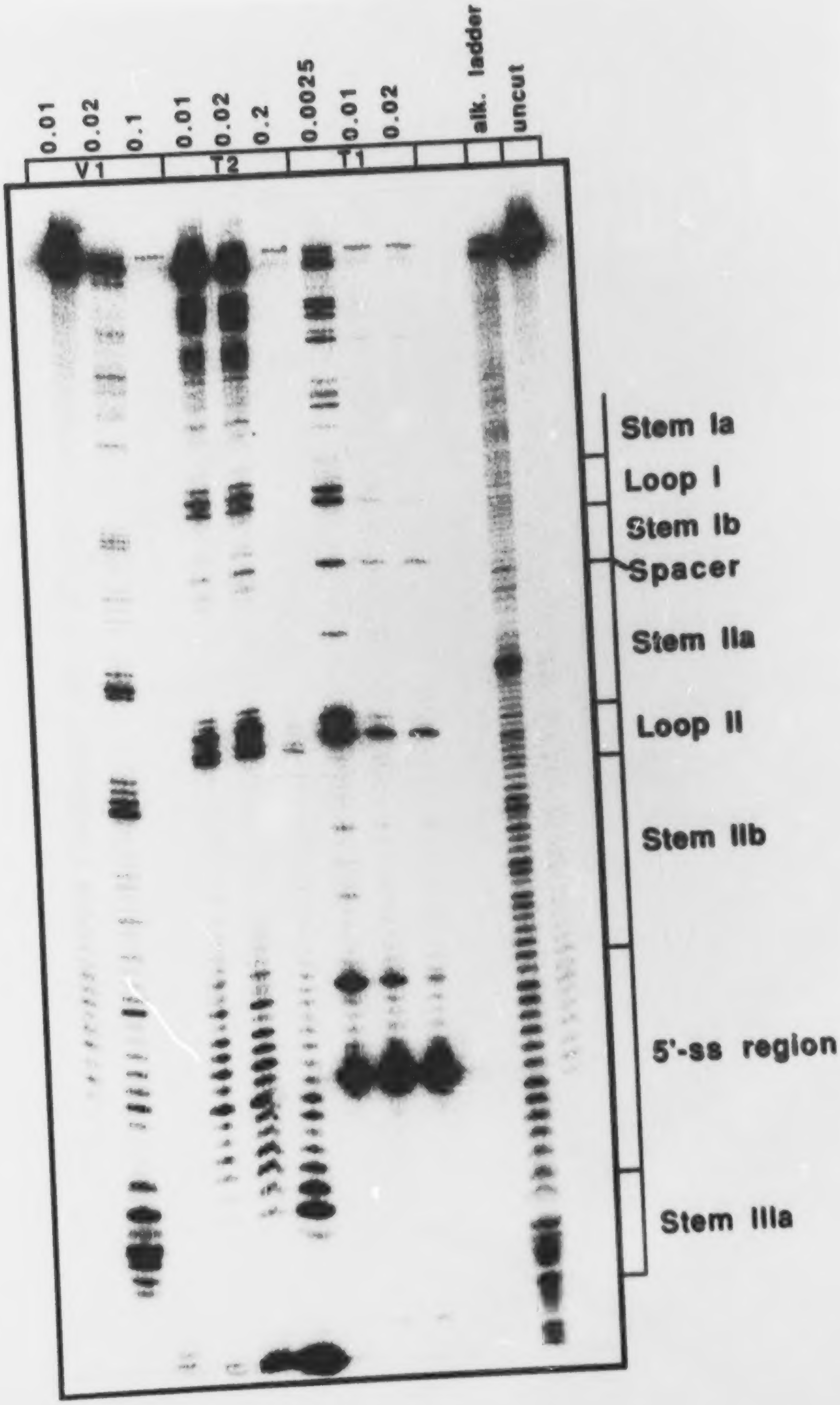
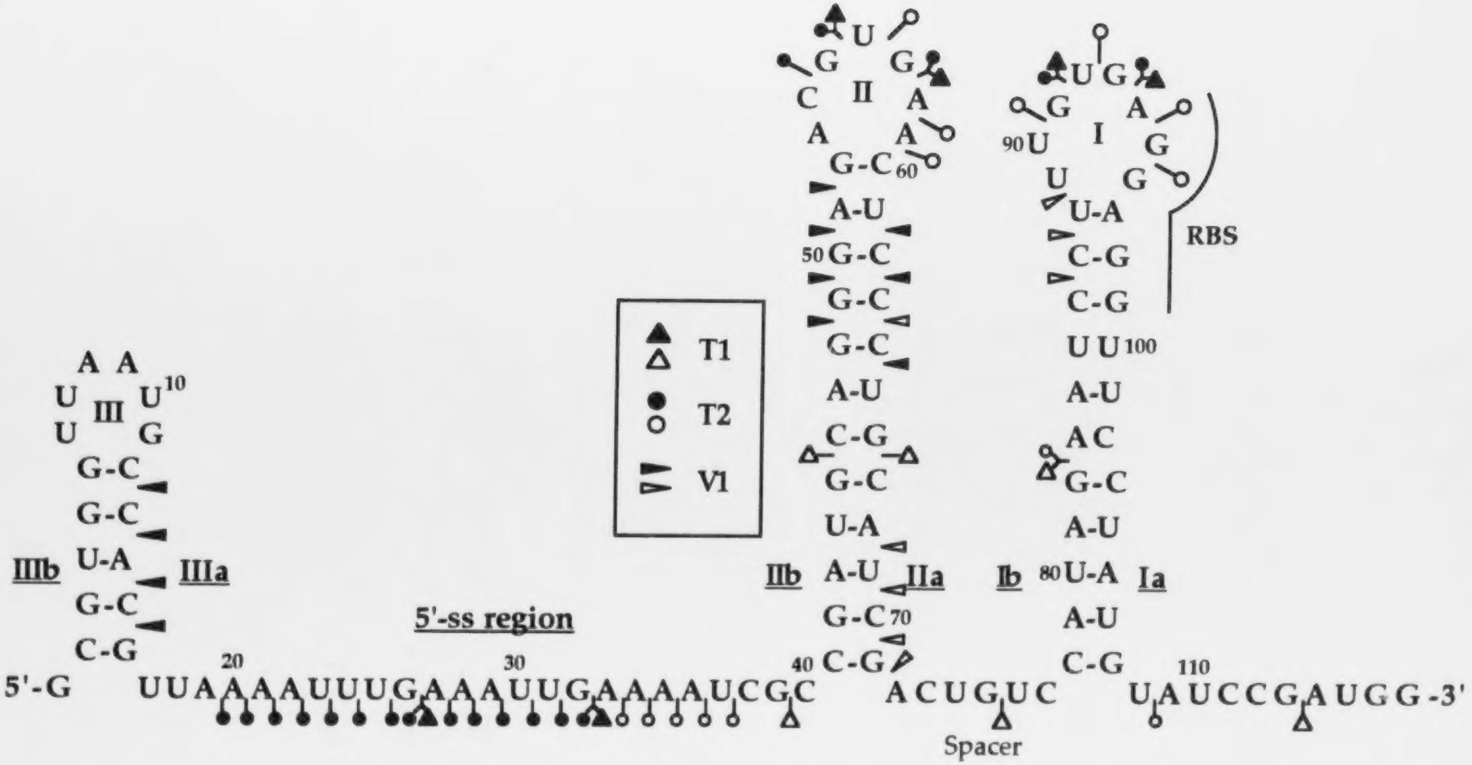

0.01
0.02
0.1
0.01
0.02
0.2
0.0025
0.01
0.02
alk. ladder
uncut
V1
T2
T1
Stem Ia
Loop I
Stem Ib
Spacer
Stem IIa
Loop II
Stem IIb
5'-ss region
Stem IIIa

Figure 3c: ii. Secondary structure of TraJ211 RNA. The graphical representation shown here is based on previously described computer predictions, phylogenetic analysis (see text) and the nuclease digests shown in (i) and other similar experiments. The cleavage positions are represented by symbols (see inset): open symbols indicate weak cleavages while solid symbols indicate strong cleavages. The nucleotide positions are numbered as in Figure 1.


T1
T2
V1
III
IIIb
IIIa
5'-ss region
II
IIb
IIa
I
Ib
Ia
RBS
Spacer
5'-G UUAAAAUUUGAAAUUGAAAAUCGC ACUGUC UAUCCGAUGG-3'
10
20
30
40
50
60
70
80
90
100
110

These results are supported by the observation that RNase T2 cleaves at every position between A_{19} and C_{37}, indicating that this entire region is single-stranded.

The stem-and-loop proximal to the 5'-end of the transcript is referred to as stem-loop II since it is complementary to FinP stem-loop II. The stem region is easily identified due to a lack of digestion by T1 and T2, while V1 cleaves at several positions on both sides of the stem. Similarly, loop II is cleaved at both G_{55} and G_{57} by T1 and at C_{54} to A_{59} by T2, demonstrating the unpaired nature of this region.

Although the resolution of the gel decreases significantly, it remains possible to identify a T1 cleavage site in the spacer (G_{75}) and several V1 cleavage sites on the 5'-side of stem I. Loop I is easily identified due to the strong cleavages of G_{91} and G_{93} by both T1 and T2. Finally, cleavages on the 3'-side of stem I and in the 3'-tail are either weak or undetectable.

III. Measurement of the kinetics of FinP - TraJ211 duplex formation

The kinetics of duplex formation between FinP and TraJ RNA were measured *in vitro*. Radioactively-labeled FinP RNA was incubated with a molar excess of unlabeled TraJ211 RNA in binding buffer. Samples were taken at intervals and analysed by electrophoresis in a denaturing polyacrylamide gel. It has previously been shown that an RNA duplex does not dissociate in the presence of 7 M urea unless the complex is heated prior to analysis (Persson *et al*, 1988) and can be separated from unpaired RNA using denaturing conditions.

The stable RNA duplex appeared as a band that was separate from those corresponding to the substrate RNAs (data not shown). The position of free and duplexed labeled FinP RNA is shown in Fig 3d.i. The relative amount of non-duplexed FinP RNA was plotted against time in Fig. 3d.ii which shows that duplex formation followed pseudo-first-order kinetics.

Figure 3d: Determination of the rate constant of duplex formation between FinP and TraJ211 RNA.

i. ^{32}P-labeled FinP RNA (5.3×10^{-11} M) was coincubated with two different concentrations of unlabeled TraJ211 RNA at 37°C. (i) [TraJ211] = 2.5×10^{-9} M. (ii) [TraJ211] = 5.0×10^{-9} M. Samples were withdrawn and diluted with an equal volume of gel loading buffer at the indicated time points and electrophoresed on an 8% polyacrylamide gel containing 7 M urea.

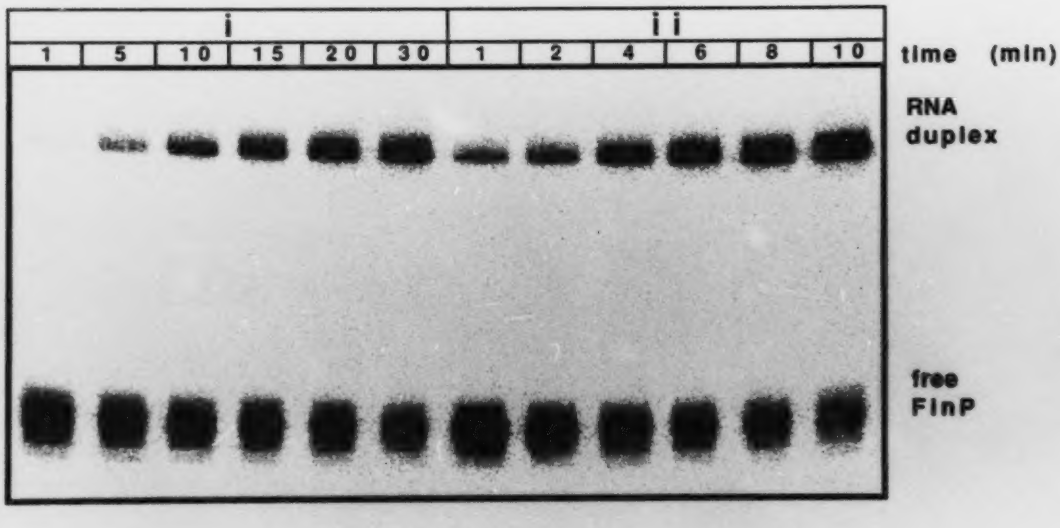
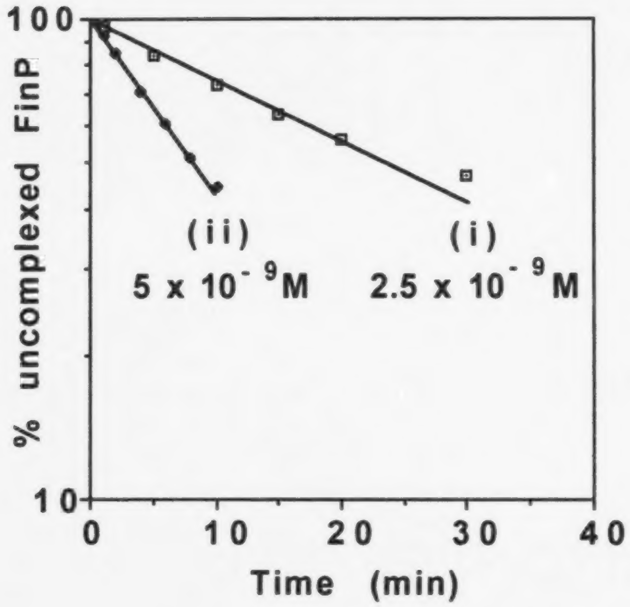

i
ii
1
5
10
15
20
30
1
2
4
6
8
10
time (min)
RNA duplex
free FinP

Figure 3d: ii. The ratio of uncomplexed FinP to RNA duplex was measured using a Molecular Dynamics Phosphorimager. The amount of free FinP, as a percent of the total FinP in the reaction, is depicted as a function of time. Each curve is identified by the concentration of TraJ211 RNA.


100
10
% uncomplexed FinP
0
10
20
30
40
Time (min)
(ii)
5 x 10⁻⁹M
(i)
2.5 x 10⁻⁹M

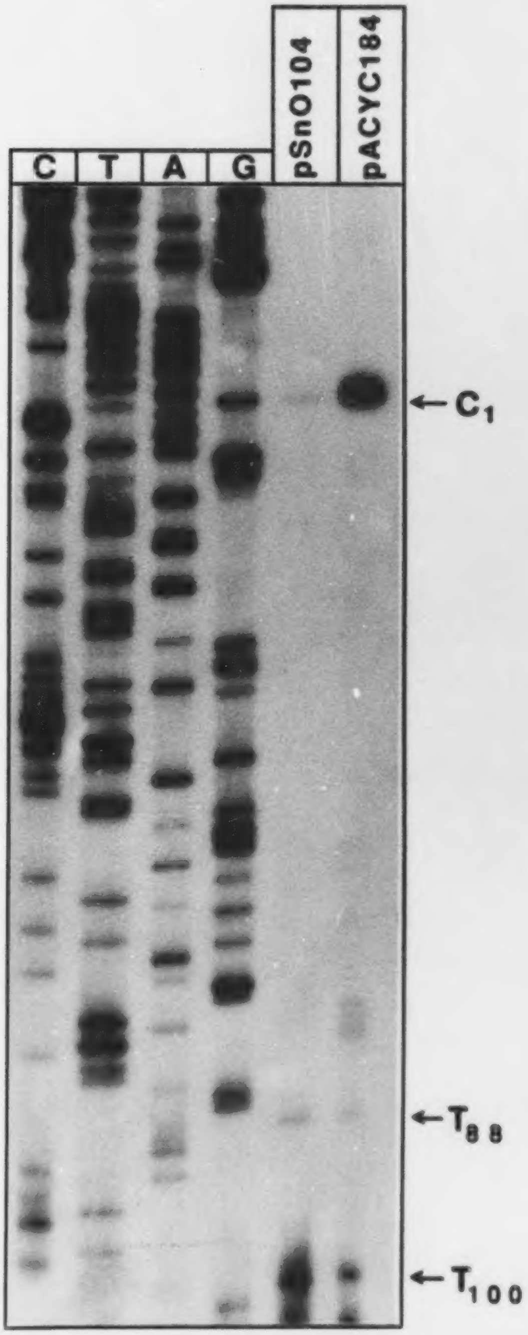
The rate doubled when the concentration of TraJ211 RNA was doubled. Hence, the reaction is bimolecular. The rate constant (k_{app}) was found to be 5 x 10^5 $M^{-1}s^{-1}$.

IV. FinOP destabilizes *traJ* mRNA *in vivo*

Primer extension analysis was used to demonstrate the effect of FinO on wild-type *traJ* mRNA expressed by the plasmid pOX38-Km, a derivative of wild type F (Fig. 3e). The plasmid pOX38-Km contains the vegetative origin of replication and the entire *tra* operon of F, and an artificially inserted kanamycin resistance gene (Chandler and Galas, 1983). Total cellular RNA was isolated from strains carrying pOX38-Km both in the presence and absence of *finO*.

In the absence of FinO, but in the presence of the control vector pACYC184, the apparently full-length *traJ* transcript is easily observed (lane pACYC184, Fig. 3e). The 5'-end of the RNA corresponds to position C_1 in Figure 3a. However, in the presence of FinO expressed from pSnO104 (Lee *et al*, 1992), there is a dramatic decrease in the amount of this extension product. At the same time, an overexposed autoradiograph revealed an additional weak band at position T_{88} while a band at position T_{100} is intensified (data not shown). The 5'-ends of these RNA species are located 24 and 12 bases upstream of the 3'-end of the putative FinP-TraJ RNA duplex, respectively.

Figure 3e: Primer extension analysis of *traJ* mRNA from pOX38/Km. RNA was analyzed from strains with (+pSnO104) and without (+pACYC184) *finO*. For the position of the TVB22 primer, see Experimental Procedures. Dideoxy sequencing reactions were carried out on plasmid DNA (pSJ99) using the same primer. Band C_1 represents the full length *traJ* mRNA transcript while T_{88} and T_{100} indicate the putative RNase III cleavage products.


C
T
A
G
pSnO104
pACYC184
←C_1
←T_{88}
←T_{100}

C. Discussion

It has previously been shown that FinO increases the half-life of FinP RNA *in vivo* in the absence of the *traJ* mRNA target (Lee *et al*, 1992). Lee *et al* (1992) also demonstrated that FinO does not have a direct effect on either the *traJ* mRNA or the *traJ* or *finP* promoters. Also, northern blotting experiments showed that FinP decreased the steady-state level of *traJ* mRNA by 3-5-fold only in the presence of FinO. FinP in the absence of FinO did not affect *traJ* mRNA concentration (Lee *et al*, 1992). In an effort to elucidate the functions of FinP and FinO, we have determined the structures of the FinP and TraJ211 RNAs *in vitro* and we have measured the effect of FinO and FinP on *traJ* mRNA *in vivo*.

Although computer predictions of the secondary structures of small RNA molecules (<100 nucleotides) can be reasonably accurate, biochemical and phylogenetic analyses can be combined with these analyses to achieve high resolution, precise structural data. A phylogenetic comparison of five alleles of *finP* reveals that most of the sequence variability is concentrated in the proposed loop regions (Finlay *et al*, 1986). In addition, genetic analysis of the *finP* gene indicates that the most detrimental mutations are localized in these same loop regions (Koraimann *et al*, 1991). This result is expected since the primary interaction between *finP* and its target is thought to occur in these structures.

The FinP secondary structure described here confirms the structures predicted by computer algorithms and genetic analysis (Koraimann *et al*, 1991; Finlay *et al*, 1986). There are two notable features of the FinP structure which differentiate it from previously well characterized antisense RNAs. First, the single-stranded regions of the molecule, other than the loops, are relatively short. Tomizawa (1984,1985) and Persson *et al* (1990a,b) have shown that unpaired stretches of RNA are required to mediate the transition from the kissing complex to the stable duplex *in vitro*. Removal of these of 27 nucleotides from the 5'-end of CopA (leaving a 7 nucleotide 5'-tail) causes a strong reduction in k_{app} of the CopA-CopT duplex (Persson *et al*, 1990a). Similarly, the loss of five nucleotides from the 5'-end of RNAI *in vivo* significantly decreases its ability to form an RNA duplex with RNAII (Tomizawa, 1984; Polisky, 1988). Finally, the lack of single-stranded regions at the 5' or 3' ends of RNA-OUT is compensated by the fact that duplex formation with RNA-IN is initiated within the single-stranded loop (Kittle *et al*, 1989). It is thus surprising that duplex formation between FinP and *traJ* mRNA occurs despite the absence of extended single-stranded termini.

Second, the sequence in FinP that is complementary to the *traJ* RBS lies partially in loop I (Fig. 3b.ii). This has not been observed in other well-characterized antisense RNA systems since CopA acts upstream of its target RBS, RNA I inhibits the function of an untranslated RNA, and RNA-OUT occludes the RNA-IN RBS only after duplex formation (Kittle *et al*, 1989).

The observed A_{12}-A_{27} mismatch in stem I of FinP indicates that there is a structural disruption of the RNA helix which prevents cleavage by the double-strand specific RNase V1. Such bulges or internal loops in the RNA helix are commonly seen in other antisense RNA molecules and are thought to facilitate the formation of the stable duplex by reducing the free energy of the stem. Alternatively, these internal loops could be important for protecting the RNA from endonucleolytic degradation by RNase III (Hjalt and Wagner, personal communication).

For the structural analysis of TraJ211 RNA, we were able to use enzymatic probing to determine the structure of the FinP target since it is located proximal to the 5'-end of the transcript (Fig. 3a). However, the sensitivity of the analysis is decreased slightly as a result of a 32-nucleotide sequence preceding the FinP target. Despite this reduction in the resolution of the analysis, it is clear that the structure of TraJ211 is essentially a complementary image of the FinP structure (compare Figures 3b and 3c). Thus the complementary loops may be involved in the formation of a kissing complex and this interaction may lead to the formation of a stable RNA duplex.

The *in vitro* FinP-TraJ211 binding experiment demonstrates that FinP is able to form a duplex with TraJ211 RNA at a rate constant (k_{app}) of about 5×10^5 $M^{-1}s^{-1}$. Similar values (usually about 10^6 $M^{-1}s^{-1}$) have been obtained for RNAI/RNAII of plasmid ColE1 (Tomizawa, 1984), CopA/CopT of plasmid R1 (Persson *et al*, 1988) and RNA-IN/RNA-OUT of IS10 (Kittle *et al*, 1989). The formation of the FinP-TraJ duplex *in vitro* occurs independently of the FinO protein, which is absolutely required for active FinP *in vivo*, suggesting that FinO has a role in the repression of *traJ* other than promoting duplex formation.

Blomberg *et al* (1990) have demonstrated the formation of the CopA-CopT duplex *in vivo* by identifying the products of cleavage by RNaseIII, an endoribonuclease that specifically cleaves double-stranded RNA molecules at a position 12-15 bp from either end of a duplex that is greater than 20 bp in length (Krinke and Wulff, 1990). RNaseIII mediated cleavage is one of several mechanisms by which antisense RNAs are thought to inactivate target mRNAs. Primer extension analysis was performed to map the 5'-end of the *traJ* mRNA *in vivo*. In the absence of FinO, an apparently full-length *traJ* mRNA is observed. However, when FinO is expressed *in trans*, the intracellular concentration of the full-length *traJ* mRNA is dramatically reduced. At the same time, two truncated species of *traJ* mRNA increase in concentration. The 5'-ends of these truncated *traJ* mRNAs are located 12 (T_{100}) and 24 (T_{88}) nucleotides from the 3'-end of the FinP-target sequence, suggesting that these may represent RNaseIII-mediated cleavage products. The appearance of these putative RNaseIII-mediated cleavage products depends on the formation of a duplex between FinP and *traJ* mRNA which, in this experiment, occurs primarily in the presence of FinO. Note that a low level of the T_{100} cleavage product is detectable in the absence of FinO, suggesting that a small amount of duplex formation occurs, presumably prior to the rapid decay of FinP.

Previous work has revealed that FinO has does not have a direct effect on the transcription of the *tra* operon (Lee *et al*, 1992), indicating that its effect is mediated through FinP. Based on the results reported here and the observations made by Lee *et al* (1992), we believe that FinP and the *traJ* mRNA have the innate ability to form a duplex both *in vitro* and *in vivo*. However, duplex formation is reduced *in vivo* due to the rapid decay of FinP RNA. Since the FinP-TraJ211 RNA duplex forms *in vitro* at a rate comparable to other antisense RNA systems, FinO appears to have a role in *traJ* mRNA repression unrelated to promoter activity or duplex formation. Thus, the primary function of FinO appears to be the stabilization of FinP in order to maintain the sufficiently high concentration of FinP required for the sequestration and inactivation of the *traJ* mRNA.

D. Materials and methods

I. Bacterial strains, plasmids, and media

pOX38-Km (Chandler and Galas, 1983) was the generous gift of K. Ippen-Ihler (Dept. of Medical Microbiology and Immunology, Texas A & M University) and was maintained in *E. coli* JC3272 (Achtman *et al*, 1971). *finO* (from plasmid R6.5) was provided *in trans* by plasmid pSnO104 (Lee *et al*, 1992). The sequence shown in Fig. 3e was generated using pSJ99 (Lee *et al*, 1992) as a template. Cells were grown in TCS broth supplemented with 25 $\mu g\ ml^{-1}$ of the appropriate antibiotics.

II. Enzymes

All restriction and modification enzymes were purchased from Pharmacia unless otherwise indicated. T7 polymerase was a gift of Leif Kirsebom and Taq polymerase was purchased from Perkin-Elmer.

III. RNA isolation and Primer extension analysis

RNA was isolated from mid-log cultures (OD_{600} of 0.75) using a modified hot phenol method as described (Frost et al. 1989).

Primer extensions were performed as previously described (Sambrook *et al*, 1989) using primer TVB22 (5'-dCCAGC AGATC TATTT GACGA GCATG), AMV reverse transcriptase (Boehringer Mannheim), and 30 μg total cellular RNA. Primer TVB22 is homologous to nucleotides 134-158 of the *traJ* sequence (numbering as in Fig. 3a). The sequence marker was generated from pSJ99, a subclone of pOX38-Km, using the TVB22 primer and Klenow enzyme (Boehringer Mannheim). The samples were electrophoresed on a 6% sequencing gel, vacuum-dried and autoradiographed on Kodak X-Omat AR film.

FinO was supplied in trans on plasmid pSnO104 (Lee *et al*, 1992) which expresses FinO from a 4 kb Pst1 fragment from plasmid R6.5 cloned into pACYC184. The vector pACYC184 was used in the FinO$^-$ control (Fig. 3e).

IV. *In vitro* transcriptions

FinP and TraJ211 RNAs were synthesized *in vitro* by run-off transcription from PCR-generated DNA templates. The FinP transcription-template was synthesized by PCR (Sambrook *et al*, 1989) from pOX38-Km to yield a fragment carrying a functional T7 promoter fused to the 79 bp FinP gene (Fig. 3a). The TraJ211 transcription-template was synthesized using pOX38-Km as a template to yield a fragment carrying a functional T7 promoter fused to the first 211 bp of the *traJ* gene (Fig. 3a). The transcriptional start sites were verified by primer extension analysis. The *in vitro* FinP RNA was identical to native FinP; the *in vitro* TraJ211 RNA was similar to the native *traJ* mRNA except that it carries two additional bases at its 5'-end, G and A respectively.

For use in the structural analyses, FinP was 5'-end-labeled with γ-^{32}P-ATP as described (Hjalt and Wagner, 1992). Uniformly labeled ^{32}P-FinP was used for the *in vitro* binding assays and was synthesized as described (Hjalt and Wagner, 1992). 'Unlabeled' TraJ211 RNA was synthesized in the presence of 100 µM α-^{3}H-UTP to allow for accurate determination of RNA concentration. All *in vitro* transcripts were gel purified as described (Persson *et al*, 1988).

V. Secondary structure analysis

Partial RNase digestion was carried out as described (Wagner and Nordström, 1986; Hjalt and Wagner, 1992) except tRNA was included at 0.4 µg/µl and RNase concentrations were determined empirically (absolute values are shown in figure legends). A size standard was generated by alkaline hydrolysis of either FinP or TraJ211 RNA by boiling ~0.1 pmol RNA in alkaline buffer (0.05 M NaOH, 1 mM EDTA) for 10 seconds. Alkaline hydrolysis was stopped by the addition of neutralizing buffer (8 M urea, 80 mM NaOAc, 1 % glacial acetic acid). Time samples were loaded immediately onto a 12% sequencing gel, dried, and visualized on a Molecular Dynamics Phosphorimager.

VI. *In vitro* FinP/TraJ211 binding assays

Binding assays were performed as described in Persson et al (1988). Visualization and quantitation of the bands were performed on a Molecular Dynamics Phosphorimager.

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Chapter 4

The FinO protein of F-like conjugative plasmids binds to, and increases the association rate of the FinP antisense RNA and its target, *traJ* mRNA

A. Introduction

Antisense RNAs are small, untranslated RNA transcripts which inhibit a wide array of cellular functions (for a recent review, see Nordström *et al*, 1993). Although most known naturally occurring antisense RNA regulatory systems have been identified in prokaryotes, an increasingly large number of eukaryotic examples have been described (Simons, 1993). In prokaryotes, these regulatory systems are most often associated with extra-chromosomal and mobile elements such as transposons, bacteriophages and plasmids. Many of the concepts derived from the study of natural antisense RNAs are being applied to the design of synthetic antisense molecules used for the inhibition of processes such as HIV replication (Homann *et al*, 1993).

Several antisense RNA-target RNA pairs have been studied in detail, most notably RNA I/RNA II which regulate plasmid ColE1 replication (Tomizawa 1984; 1990a), CopA/CopT which control plasmid R1 replication (Persson *et al*, 1988; 1990a; 1990b), and RNA-OUT/RNA-IN which regulate IS10 transposition (Kittle *et al*, 1989). A common feature of most antisense RNAs is the high degree of secondary structure exhibited by these molecules, most often in the form of one or more stem-loop domains. This feature is thought to increase the efficiency of the formation of the inhibitory antisense RNA - target RNA complex (Persson *et al*, 1990b). The kinetics of duplex formation between the two component RNAs are also similar in each case, with binding rate constants within one order of magnitude of $1 \times 10^6 M^{-1} s^{-1}$.

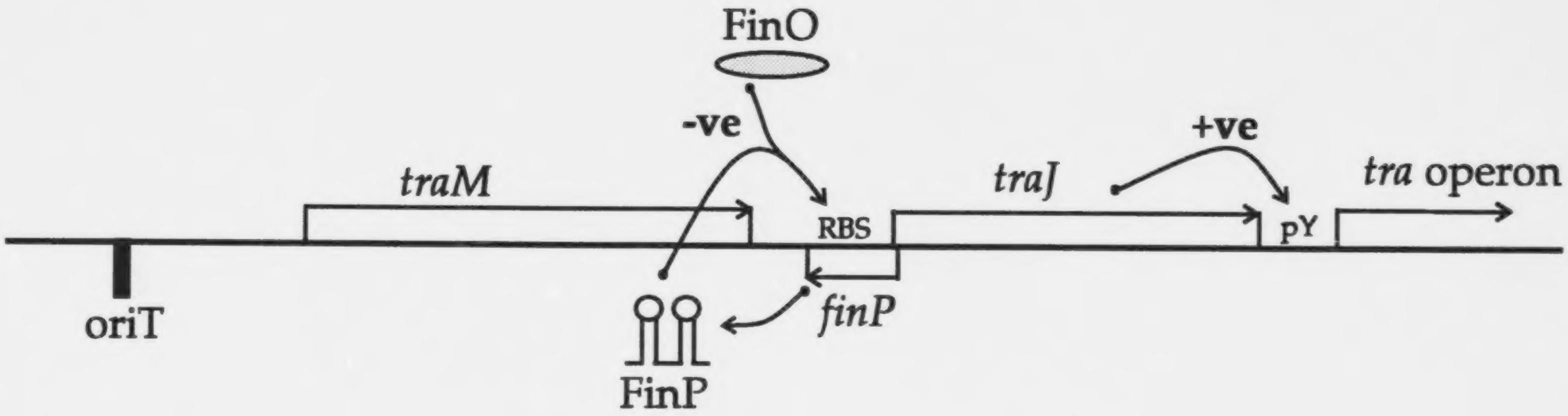
Inhibition of ColE1 replication by RNA I is unique because its effect is modulated by a small 63 amino acid protein called Rom (Tomizawa and Som, 1984). Rom is encoded immediately downstream of the replication origin and has been shown to enhance the inhibitory effect of RNA I on primer formation by more than two-fold both *in vivo* (Twigg and Sherratt, 1980) and *in vitro* (Tomizawa, 1990b; Eguchi and Tomizawa, 1990). The Rom dimer does not interact with either RNA I or RNA II independently, but rather it stabilizes the initial transient complex (the "kissing complex"), thereby favoring the formation of the stable duplex.

A second example of antisense RNA - protein interaction has been reported by Andersen and Delihas (1990). *micF* antisense RNA, which inhibits the synthesis of the OmpF protein, is bound specifically by an 80 kDa protein from *E. coli* cell extracts. The authors postulate that *micF* RNA inhibits OmpF expression as a ribonucleoprotein complex.

In this communication, we analyze the interaction between the FinO protein, the FinP antisense RNA and its target, *traJ* mRNA. This antisense RNA system decreases the rate of conjugative DNA transfer by 50-100-fold and is thus referred to as the fertility inhibition (FinOP) system. The 33 kb *tra* operon of IncF1 conjugative plasmids encodes most of the genes required for the conjugative transfer of DNA (for a recent review, see Frost *et al*, 1994). Transcription of the operon, which is under the control of the pY promoter, is activated by the product of the *traJ* gene which lies immediately upstream of pY (Fig. 4a). Expression of TraJ, and thus expression of the *tra* operon, is inhibited by the FinOP system. This inhibitory mechanism consists of two genes, *finP* and *finO*, both of which are absolutely required to mediate fertility inhibition.

finP is transcribed from the same region of DNA as, but in the opposite orientation to, the 5'-untranslated leader sequence of the *traJ* mRNA (Fig. 4a) (Dempsey, 1987). The product of *finP* is a 78 base RNA molecule (Mullineaux and Willetts, 1985) which is complementary to the 5'-untranslated region, the ribosome binding site, and the translational start codon of the *traJ* mRNA. FinP exhibits a highly ordered secondary structure consisting of two stem-loop domains and a short single-stranded 5'-tail. The 5'-untranslated region of *traJ* mRNA adopts a conformation complementary to that of FinP (van Biesen *et al*, 1993). *In vitro*, in the absence of FinO, FinP and *traJ* mRNA form a stable duplex at a rate comparable to previously characterized antisense RNA systems (van Biesen *et al*, 1993).

Figure 4a: The F *tra* operon regulatory region. Transcription of the *tra* operon from pY is activated (+ve) by the *traJ* gene product which, in turn, is negatively (-ve) regulated by the FinP antisense RNA and the FinO protein. RBS = ribosome binding site; pY = promoter for the *tra* operon; oriT = origin of DNA transfer. Drawing is not to scale.


FinO
-ve
+ve
traM
RBS
traJ
pY
tra operon
oriT
finP
FinP

In vivo, the co-expression of FinP and FinO results in a dramatic decrease in the steady-state levels of *traJ* mRNA (Lee *et al*, 1992; van Biesen *et al*, 1993). Similarly, FinO and FinP decrease the -galactosidase activity of a *traJ-lacZ* fusion by more than 1000-fold (Gaffney *et al*, 1983; van Biesen and Frost, unpublished data). It has been suggested that this effect on *traJ* mRNA may be due to cleavage of the FinP-*traJ* mRNA duplex by the host encoded RNase III (van Biesen *et al*, 1993)

The *finO* gene is located at the distal end of the *tra* operon and is not under the transcriptional control of the pY promoter. FinO is a highly basic 21.2 kDa protein and its level of expression regulates the efficiency of fertility inhibition (van Biesen and Frost, 1992). Lee *et al* (1992) have demonstrated that FinO increases the half-life of FinP *in vivo* from 3 min to more than 40 min in the absence of the *traJ* mRNA target, suggesting that FinO physically protects FinP from ribonucleolytic degradation.

Here we report the interaction of a protein with an antisense RNA and its target and its effect on antisense RNA - target RNA duplex formation. The data show that FinO is able to bind with a moderate affinity to the fully paired second stem domain of FinP and its complement in *traJ* mRNA. Also, the stable FinP-*traJ* mRNA duplex is bound by FinO in at least two positions. Analysis of the kinetics of FinP-*traJ* mRNA duplex formation reveals that FinO increases the rate of formation of the duplex by almost a factor of 5. A model of FinOP mediated inhibition of TraJ expression is proposed and a comparison between the ColE1 Rom protein and FinO is presented.

B. Results

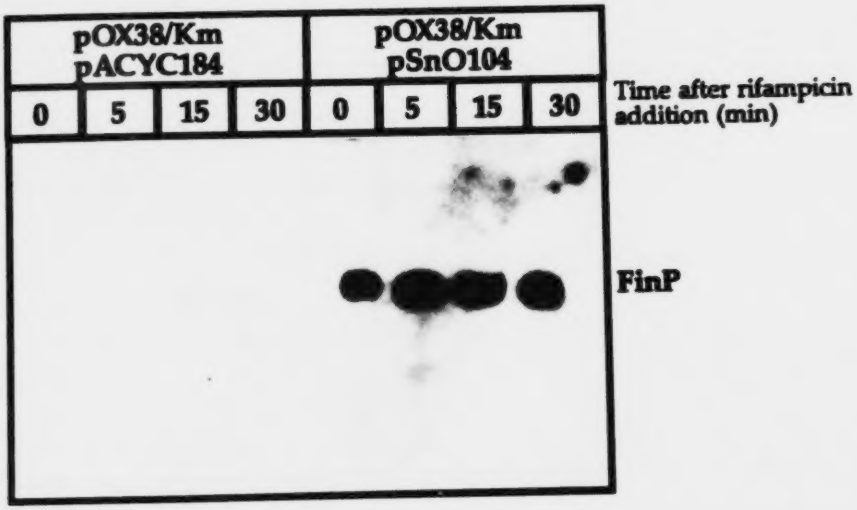
I. FinO protein stabilizes the FinP antisense RNA *in vivo*

The ability of FinO to extend the chemical and functional half-life of a lacZ-FinP RNA transcriptional fusion has previously been shown (Lee *et al*, 1992). Using Northern blotting analysis, FinO expressed *in trans* was shown to increase the steady-state level of wild-type FinP expressed from the F plasmid (Fig. 4b). In this experiment, FinP was expressed from the low copy number F plasmid derivative, pOX38-Km, which is *finO*$^-$ (Chandler and Galas, 1983). FinO derived from plasmid R6-5 was expressed, *in trans*, by the chimeric plasmid pSnO104 (Lee *et al*, 1992). In the absence of FinO, FinP RNA was not detectable, suggesting that it was turned over rapidly. However, when FinO was expressed *in trans*, the amount of FinP RNA increased dramatically and remained unchanged, even in the presence of added rifampicin to inhibit further initiation of transcription. This prolonged half-life of FinP RNA may be the result of either an inhibitory effect of FinO on a FinP-specific ribonuclease, or a direct physical association between FinO and FinP which sterically prevents cleavage by a ribonuclease. To differentiate between these possibilities, we examined the ability of purified FinO to interact with FinP *in vitro*.

II. FinO binds to both FinP and *traJ* mRNA

Attempts to purify FinO protein from cultures over-expressing the *finO* gene were severely hindered by its propensity to form insoluble aggregates (van Biesen and Frost, unpublished data). The entire coding region of the *finO* gene was cloned into pGEX-2T, a vector designed to express glutathione-S-transferase (GST) fusion proteins from the IPTG (isopropyl--D-thiogalactopyranoside) inducible *tac* promoter. After induction, the GST-FinO protein was purified to >95% purity using a novel method of glutathione-agarose affinity purification (Frangioni and Neel, 1993).

Figure 4b: Northern analysis of FinP RNA in the absence (pACYC184) and presence (pSnO104) of a co-resident chimeric plasmid expressing FinO. Cells containing the indicated plasmids were grown to mid-log and treated with rifampicin to inhibit further transcription. Samples were taken at the times indicated after the addition of rifampicin. Total cellular RNA was electrophoresed on an 8% 8 M urea polyacrylamide gel and probed for the presence of FinP RNA (see Materials and methods).


pOX38/Km
pACYC184
pOX38/Km
pSnO104
0
5
15
30
0
5
15
30
Time after rifampicin addition (min)
FinP

Attempts to remove the GST moiety of the fusion by thrombin cleavage were unsuccessful, perhaps due to inaccessibility of the thrombin cleavage site. As a result, all negative control experiments were done in the presence of purified GST protein.

The pGEX-FO2 construct was assayed for FinO activity *in vivo* using a standard mating assay. Lee *et al* (1992) have shown that wild-type FinO reduces the mating efficiency of F to 1% of its wild-type levels. Our results revealed that the rate of conjugal transfer of F is not affected by the presence of the vector, pGEX-2T, in the cell. However, the presence of the fusion protein expressed by pGEX-FO2 caused a decrease in the mating efficiency of F to 3-3.5 % of its wild-type levels, indicating that the GST-FinO fusion retains a measurable amount of FinO function. Since the intracellular concentration of the GST-FinO protein is not known, it is not possible to directly compare the activity of this fusion to the wild-type FinO protein.

FinP and *traJ* mRNA transcripts were synthesized *in vitro* from DNA templates generated by PCR (van Biesen *et al*, 1993). A truncated form of *traJ* mRNA, TraJ211, was used in lieu of the full length *traJ* mRNA transcript. TraJ211 RNA, which consists of the first 211 nt of the wild-type *traJ* mRNA, has been shown to form a duplex with FinP with normal binding kinetics (van Biesen *et al*, 1993).

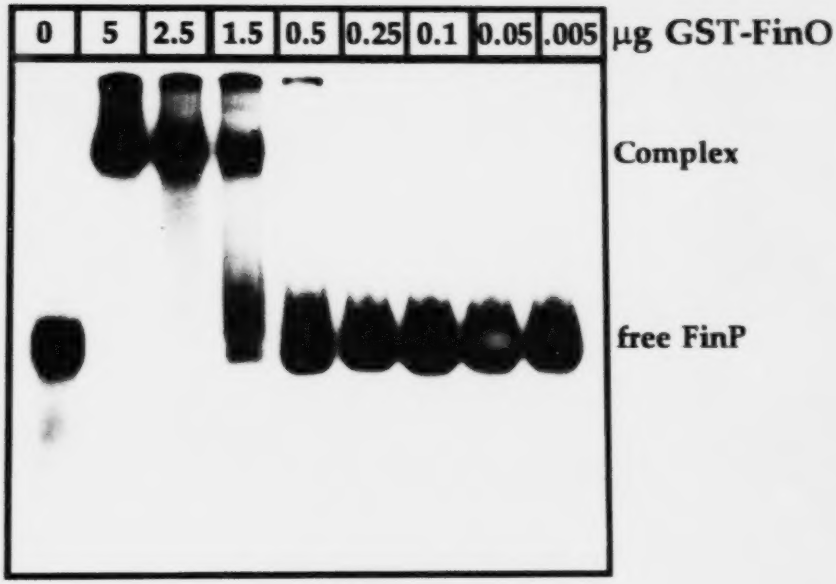
Co-incubation of a large molar excess of purified GST-FinO with radioactively labeled FinP RNA caused a shift in the mobility of the FinP band on a non-denaturing polyacrylamide gel (Fig. 4c.i). The amount of ^{32}P-FinP shifted was dependent on the concentration of the GST-FinO in the binding mixture. The association equilibrium constant (K_a) was calculated using the GST-FinO concentration which caused 50% of the labeled RNA to be retarded (Tsai *et al*, 1990; see Materials and Methods for calculations). For the binding of GST-FinO to ^{32}P-FinP, K_a was $\sim 4 \times 10^5$ M^{-1}. Since the proportion of active protein in the GST-FinO preparation was not known, this value may be artificially low. Reciprocal experiments suggested that the fraction of functional GST-FinO in the concentrated preparations was high, but that dilution of the fusion

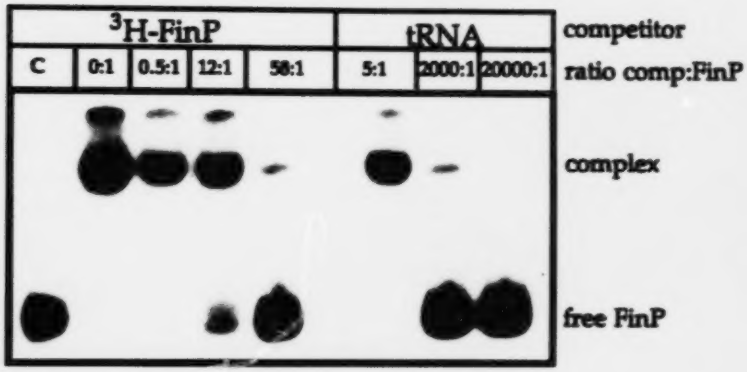
Figure 4c: **i)** Mobility shift assay of FinP RNA by GST-FinO.

FinP RNA (~5 x 10^{-10} M) was co-incubated for 30 min at room temperature with the indicated amounts of purified GST-FinO fusion protein. Samples were loaded on a continuously running 8% non-denaturing polyacrylamide gel. The locations of the free and bound ^{32}P-FinP are indicated. Densitometric scans of this and similar gels were used for the determination of the association equilibrium constant (K_a).

ii) The specificity of GST-FinO binding.

Binding of GST-FinO to FinP RNA was carried out in the presence of various amounts of the indicated unlabeled specific and non-specific competitor RNAs. Labeled and unlabeled RNAs were combined prior to the addition of GST-FinO. Reaction and electrophoresis conditions are identical to those described in Fig. 4c.i. The locations of the free RNA and the GST-FinO complex are indicated.


0
5
2.5
1.5
0.5
0.25
0.1
0.05
.005
μg GST-FinO
Complex
free FinP


^{3}H-FinP
tRNA
competitor
C
0:1
0.5:1
12:1
58:1
5:1
2000:1
20000:1
ratio comp:FinP
complex
free FinP

protein led to a disproportionately large decrease in the amount of FinP bound. Also, it is not known whether GST-FinO binds in a monomeric or multimeric form, although it appeared to exist as a monomer in solution as demonstrated by gel exclusion chromatography (data not shown). In a control experiment, the incubation of ^{32}P-FinP with GST alone did not result in mobility shift of the RNA (data not shown). The FinO-mediated shift was abolished by the addition of excess unlabeled FinP, but was unaffected when similar amounts of a non-specific competitor, tRNA, were added to the reaction (Fig. 4c.ii). Binding was still detectable in the presence of a 2000-fold excess of tRNA, indicating that the FinO-FinP interaction was specific.

Similar results were obtained when FinO was co-incubated with TraJ211 RNA (Fig. 4d.i). The mobility of the ^{32}P-TraJ211 band was reduced in the presence of GST-FinO and the calculated value of K_a was similar to that for FinP ($K_a = \sim 4 \times 10^5$ M^{-1}). A GST-FinO - ^{32}P-TraJ211 complex was not visible in lane 1 (4 g GST-FinO) because of aggregation of the reaction components, preventing the ^{32}P-TraJ211 from entering the gel. Formation of the GST-FinO - ^{32}P-TraJ211 complex was inhibited by the addition of unlabeled TraJ211 but to a lesser extent by an excess of tRNA (Fig. 4d.ii), demonstrating the specificity of this protein-RNA interaction.

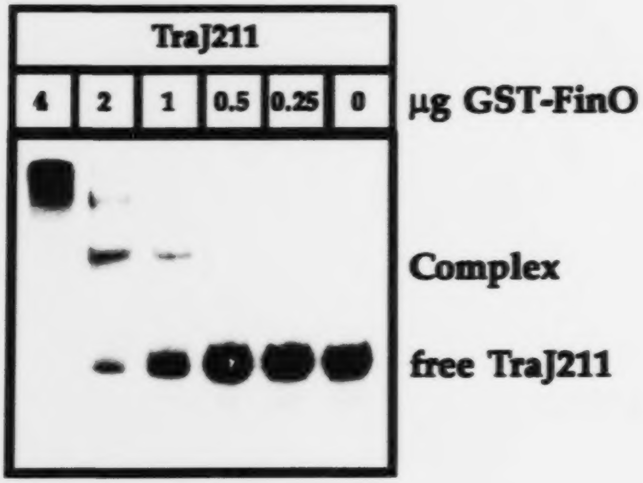
These data demonstrate that the GST-FinO protein bound in a specific manner to both FinP and TraJ211. Previously, it has been demonstrated that stem-loop II of FinP and its complementary stem-loop in TraJ211 exhibit almost identical secondary structures in the stem domain, whereas stem-loop I and its complement are significantly different (van Biesen *et al*, 1993). Based on these data, we postulated that the FinO binding site may be located on stem-loop II of FinP and not stem-loop I.

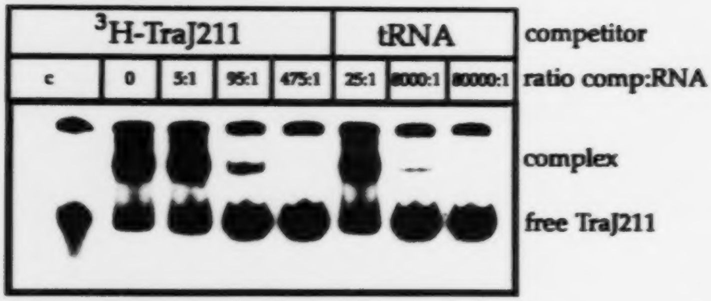
Figure 4d: **i)** Mobility shift assay of TraJ211 RNA by GST-FinO.

TraJ211 RNA (~2 x 10^{-10} M) was co-incubated for 30 min at room temperature with the indicated amounts of purified GST-FinO fusion protein. Samples were loaded on a continuously running 8% non-denaturing polyacrylamide gel. The locations of the free and bound ^{32}P-TraJ211 are indicated. Densitometric scans of this and similar gels were used for the determination of the association equilibrium constant (K_a).

ii) The specificity of GST-FinO binding.

Binding of GST-FinO to TraJ211 RNA was carried out in the presence of various amounts of the indicated unlabeled specific and non-specific competitor RNAs. Labeled and unlabeled RNAs were combined prior to the addition of GST-FinO. Reaction and electrophoresis conditions are identical to those described in Fig. 4d.i. The locations of the free RNA and the GST-FinO complex are indicated.


TraJ211
4
2
1
0.5
0.25
0
μg GST-FinO
Complex
free TraJ211


^{3}H-TraJ211
tRNA
competitor
c
0
5:1
95:1
475:1
25:1
8000:1
80000:1
ratio comp:RNA
complex
free TraJ211

III. FinO binds to stem-loop II of FinP

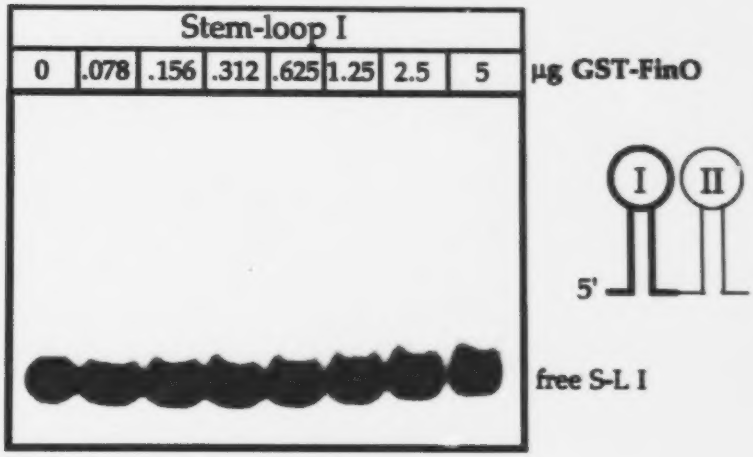
To determine the location of the FinO binding site on FinP RNA, we synthesized each of the two stem-loop domains of FinP from separate PCR templates. GST-FinO, when present in a large molar excess, did not affect the mobility of stem-loop I of FinP (Fig. 4e.i). In contrast, GST-FinO bound to stem-loop II RNA with an association equilibrium constant similar to that calculated for the full length FinP RNA ($K_a = \sim 1.5 \times 10^6 M^{-1}$) (Fig. 4e.ii), indicating that the site of FinO-FinP interaction is located on the second stem-loop domain of FinP.

The observation that GST-FinO bound to FinP and TraJ211 equally well suggested that the loop II domain was not involved in binding, since the sequences of the FinP and TraJ211 loop II domains are complementary and are therefore likely to adopt different structures. Secondary structure analysis of the stem II domains of FinP and *traJ* mRNA (van Biesen *et al*, 1993) revealed that this structure is fully double-stranded along its entire length. Due to the complementarity of these domains, the fully paired FinP-*traJ* mRNA duplex was predicted to contain two such FinO binding sites.

IV. FinO binds to at least two sites on the FinP-TraJ211 duplex

^{32}P-FinP and unlabeled TraJ211 were heated and renatured slowly at room temperature to form the ^{32}P-FinP-TraJ211 duplex. The labeled duplex was gel purified and co-incubated with increasing concentrations of GST-FinO (Fig. 4f). As the concentration of GST-FinO increased, the mobility of the duplex was retarded in two or more discrete increments. Complex C1 represents a single shift in the mobility of the duplex RNA, indicating that it was bound at a single site by GST-FinO. However, in the presence of increased concentrations of FinO, the mobility of the labeled duplex was further retarded (complex C2), suggesting that the fully formed FinP-TraJ211 duplex contained at least two binding sites for GST-FinO.

Figure 4e: Mobility shift assay of FinP stem-loops I and II.
Binding of GST-FinO to stem-loop I (panel i) and stem-loop II (panel ii) was assayed using the indicated amounts of protein. The reaction and electrophoresis conditions are identical to the experiment in Fig. 4d except the polyacrylamide gel is 12%. The locations of the free and complexed RNA are shown. The region of FinP used in each binding assay is shown in bold in the accompanying diagrams.


Stem-loop I
0
.078
.156
.312
.625
1.25
2.5
5
µg GST-FinO
I
II
5'
free S-L I

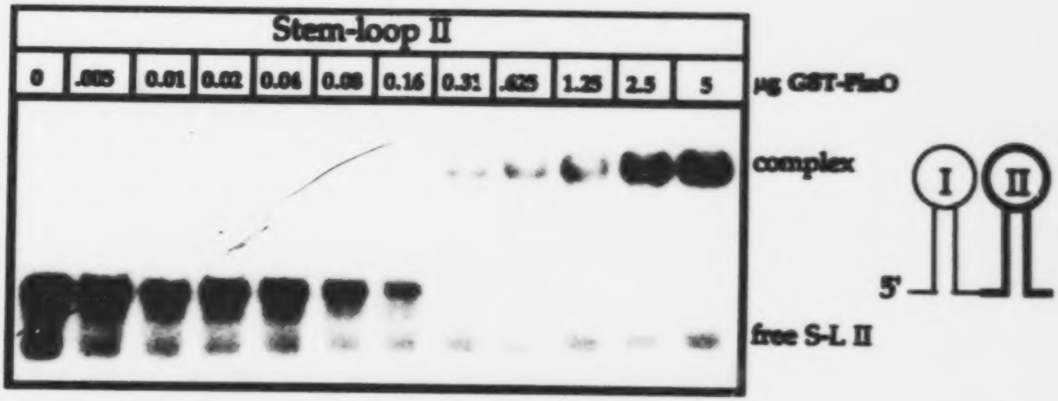
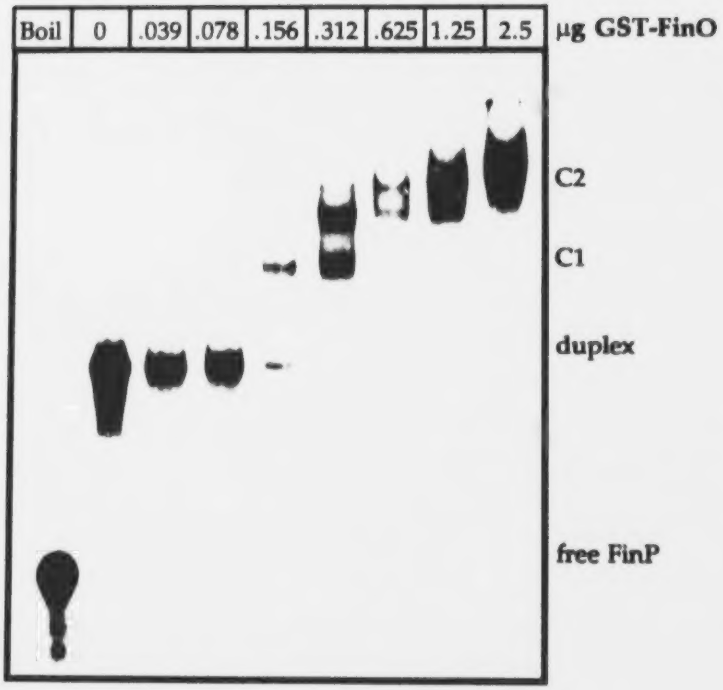

Stem-loop II
0
.005
0.01
0.02
0.04
0.08
0.16
0.31
.625
1.25
2.5
5
µg GST-FinO
complex
I
II
5'
free S-L II

Figure 4f: Mobility shift assay of the FinP-TraJ211 duplex RNA. Binding of GST-FinO to the FinP-TraJ211 duplex was assayed using the indicated amounts of protein. The reaction and electrophoresis conditions are identical to those described in Fig. 4d. The locations of the free FinP, the unbound FinP-TraJ211 duplex and the two GST-FinO/duplex complexes (C1 and C2) are indicated. Densitometric scanning of this autoradiograph was used to determine the relative dissociation constants of complexes C1 and C2.


Boil
0
.039
.078
.156
.312
.625
1.25
2.5
µg GST-FinO
C2
C1
duplex
free FinP

The relative dissociation constants of the two complexes (Kd_{C1} and Kd_{C2}) could be determined based on the fractional maximum level ($fmax_{C1}$) of complex C1 measured in the binding reactions (Tsai *et al*, 1989; see Materials and methods for calculations). Densitometric scans of the autoradiograph shown in Fig. 4f revealed that complex C1 contained at least 40% of the total amount of ^{32}P-TraJ211 in the reaction. The relative dissociation constant of complex C2 (Kd_{C2}) was calculated to be only 2.25 -fold less than Kd_{C1}, suggesting that the binding of FinO to the duplex is non-cooperative and that these two FinO sites are bound with equal affinities.

The presence of what may be additional complexes (the bands above complex C2, Fig. 4f) at high concentrations of GST-FinO may be due to either protein-protein interactions or low-specificity cooperative binding of GST-FinO. This effect may be an artifact due to the high concentration of protein in the binding reaction, or an actual FinO-FinP binding pattern required for the regualtion of *traJ*. These results suggest that FinO interacts in a specific manner with the double-stranded stem-loop II domain of FinP RNA and its complement in *traJ* mRNA .

V. FinO increases the rate of FinP-TraJ211 duplex formation

The *in vitro* kinetics of duplex formation between FinP and TraJ211 have previously been measured (van Biesen *et al*, 1993). Duplex formation was found to be a bimolecular reaction that progressed at a rate similar to those reported for other naturally occurring antisense RNA systems. To test the effect of FinO on the kinetics of FinP-*traJ* mRNA duplex formation, we have repeated the analysis of the binding kinetics in the presence of a molar excess of GST and GST-FinO protein (Fig. 4g.i). In this experiment, the reaction buffer was altered slightly to allow duplex formation and to favor interaction of the protein with the RNA (see Materials and methods). The composition of the buffer previously used for the analysis of the binding kinetics

Figure 4g: The effect of FinO on the rate constant of FinP-TraJ211 duplex formation. i. ^{32}P-labeled FinP RNA (~5 x 10^{-10} M) was coincubated with 5 x 10^{-9} M unlabeled TraJ211 RNA and GST (3.3 x 10^{-6} M) or GST-FinO (1.8 x 10^{-6} M) at 37°C. Samples were taken at the indicated time points and diluted in 2x formamide loading buffer prior to loading on an 8% non-denaturing polyacrylamide gel.

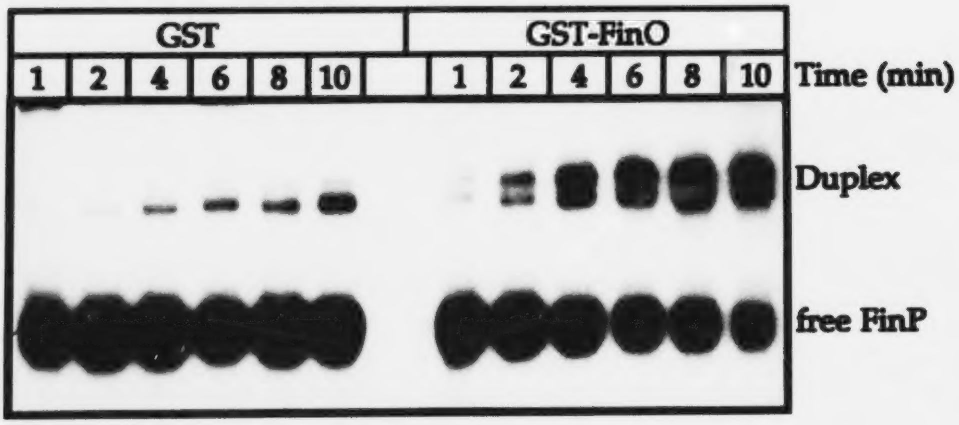
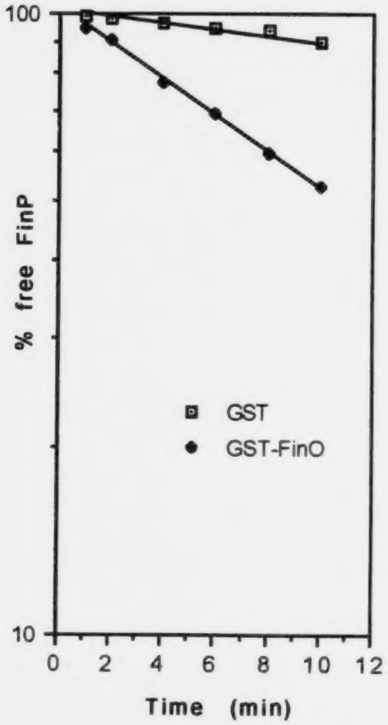

GST
GST-FinO
1
2
4
6
8
10
1
2
4
6
8
10
Time (min)
Duplex
free FinP

Figure 4g: ii. Graphical representation of the experiment in panel i. Densitometric scanning of the autoradiograph was used to determine the relative amounts of free and complexed FinP. The amount of free FinP, as a percent of the total FinP in the reaction, is depicted as a function of time. The k_{app} values of each reaction are described in the text.


100
10
% free FinP
0
2
4
6
8
10
12
Time (min)
GST
GST-FinO

did not favor protein-RNA interactions due to the high salt concentration and the absence of BSA. As a result, the calculated rate constants of duplex formation were not directly comparable to previously reported values (van Biesen *et al*, 1993).

Furthermore, the reaction products were analyzed on a non-denaturing polyacrylamide gel since electrophoresis in the presence of 8 M urea resulted in the formation of protein-RNA aggregates which were retained in the sample loading wells. The inability to detect a FinP-*traJ* mRNA duplex on a denaturing gel in the presence of GST-FinO suggests that this species may in fact not form. It is reasonable to propose that the complex formed between FinP and TraJ211 RNA in this experiment (Fig. 4g.i) is not a stable duplex, but rather a stable intermediate similar to the kissing intermediate proposed by Tomizawa (1984). We have not yet determined the ratio of the components present in this electrophoretically retarded species.

GST-FinO increased the rate of FinP-TraJ211 duplex formation (Fig. 4g.i). ^{32}P-FinP was incubated in the presence of a molar excess of TraJ211 (4.6×10^{-9} M) and samples were taken at various times and electrophoresed on an 8% polyacrylamide gel. The rate of disappearance of free ^{32}P-FinP was plotted as a function of time (Fig. 4g.ii). The apparent second order rate constant of duplex formation (k_{app}) in the presence of GST (3.3×10^{-6} M) was calculated to be $\sim 5 \times 10^{4}$ M^{-1}s^{-1}. In contrast, the addition of GST-FinO (1.8×10^{-6} M) to the duplexing reaction increased the k_{app} to $\sim 2.4 \times 10^{5}$ M^{-1}s^{-1}, an increase of almost 5-fold. Thus, the presence of the FinO moiety of the GST-FinO fusion appeared to promote the formation of the FinP-TraJ211 duplex *in vitro*, increasing the rate of duplex formation by a factor of almost 5-fold.

C. Discussion

These results suggest a direct interaction between both members of the FinP-*traJ* mRNA antisense RNA system with a protein, FinO. This interaction involves the binding of FinO to both the FinP antisense RNA and its target, *traJ* mRNA. In addition to increasing the chemical and functional stability of FinP, FinO appears to increase the rate of formation of the stable FinP-*traJ* mRNA duplex.

Lee *et al* (1992) have demonstrated that FinO increases the chemical stability of FinP expressed from a high copy-number vector *in vivo* by more than 15-fold. Preliminary evidence suggested that this may be due to the inhibition of the degradation of FinP by the host RNase E endoribonuclease. Northern analysis of FinP expressed from a low copy number derivative of F, pOX38-Km, indicated that FinP was detectable only when FinO was expressed *in trans*, suggesting that FinO inhibited cleavage of the RNA by a ribonuclease.

The ability of FinO to protect the structure of FinP *in vivo* suggests that there is a direct interaction between these two molecules. The chimeric plasmid, pGEX-FO2, which expressed a GST-FinO fusion from an IPTG inducible *tac* promoter, retained almost complete FinO function, as evidenced by its ability to repress transfer of the F plasmid *in vivo* to 3% of its wild-type levels. It is difficult to provide a direct comparison of the activity of GST-FinO with the native FinO since the intracellular levels of the proteins are not known. Electrophoretic mobility shift assays of FinP RNA using purified GST-FinO indicated that the FinO moiety of the fusion protein bound to FinP antisense RNA with a moderate affinity ($K_a = 4 \times 10^5$ M^{-1}). Whether GST-FinO bound to FinP as a monomer or a multimer has not been determined, although the fusion was a monomer in solution as determined by gel exclusion chromatography. The binding between FinO and FinP was shown to be specific as assayed by competition analysis, where unlabeled FinP inhibited the FinO-mediated mobility shift of FinP more effectively than the non-specific competitor, *E. coli* tRNA.

TraJ211 RNA was bound by GST-FinO with almost the same affinity as FinP. This unexpected result can be explained by comparison of the FinP and TraJ211 secondary structures (van Biesen *et al*, 1993). Although the sequences of FinP and TraJ211 are complementary along their entire lengths, they share only one truly similar structure, the stem domain of stem-loop II, which is devoid of bulges, mismatches or G-U base pairs. Mismatched base pairs in stem-loop I result in the formation of slightly different secondary structures in the antisense and target RNAs. Thus, the stem II domain of each RNA was predicted to contain a FinO binding site. To test this hypothesis, the two FinP stem-loop domains were synthesized separately *in vitro* and assayed for their interaction with GST-FinO as measured by gel retardation.

GST-FinO interacted with the second stem-loop domain of FinP but not with stem-loop I. This interaction occurred with an association equilibrium constant ($K_a = 1.5 \times 10^6 \ M^{-1}$) comparable to the full length FinP RNA, suggesting that all of the determinants required for FinO binding are located on this structure. It is unlikely that FinO binds to the loop domain of stem-loop II since the sequences of this loop differ between FinP and *traJ* mRNA and between various alleles of FinP (Finlay *et al*, 1986). Whereas Rom has been shown to bind only to the interacting loops of RNA I and RNA II (Tomizawa and Som, 1984), FinO appears to interact directly with the stem domains of both FinP and *traJ* mRNA, suggesting that it binds non-specifically to all regions of extended double-stranded RNA.

If the FinO binding sites on FinP and *traJ* mRNA are indeed located on the double-stranded stem domain, then the formation of a stable FinP-*traJ* mRNA duplex should result in the formation of at least two such binding sites on the same molecule. A mobility shift assay of the stable duplex by GST-FinO resulted in the detection of both these binding sites, as shown by the appearance of two discrete reatrded duplex-containing complexes. As the concentration of GST-FinO was increased, the TraJ211

RNA was shifted to an intermediate position (complex C1), probably due to the occupation of a single FinO binding site by GST-FinO. At higher protein concentrations, a second larger complex appeared (complex C2), representing the duplex on which two FinO binding sites are occupied. Comparison of the relative dissociation constants suggested that there was not a significant difference (~2-fold) between the stability of these two complexes, suggesting that the binding of GST-FinO to the duplex was not cooperative. In the presence of a large excess of GST-FinO, a third and fourth complex are detectable, perhaps as a result of non-specific protein-protein interactions or low-specificity cooperative binding of GST-FinO.

These results indicated that GST-FinO binds to a region of A-form double-stranded RNA in a sequence-specific manner. Most RNA binding proteins appear to interact with specific secondary structures, such as mismatches, bulges in stem domains or single-stranded loops (Mattaj, 1993). These structures allow the protein to recognize salient conformational alterations in the RNA molecule. For example, mutational analysis of the stem I domain of RNA I from plasmid ColE1 suggests that a portion of the Rom binding site consists of an unmatched C residue near the top of the stem (Moser *et al*, 1984). In double-stranded regions of A-form RNA, the functional groups of bases are buried in the narrow major groove of the helix, thereby inhibiting specific protein-RNA interactions. Thus the apparent specificity of binding of FinO to the stem-loop II domain of FinP and its complement in *traJ* mRNA is unusual. This specificity may be mediated by sequence-specific alterations of the double-stranded nature of the stem, or by direct protein-RNA contacts in the major groove of the helix. This type of interaction may occur if the amino acids of FinO which contact the RNA are in an extended conformation or perhaps a tight alpha-helix, although this type of binding has not yet been observed.

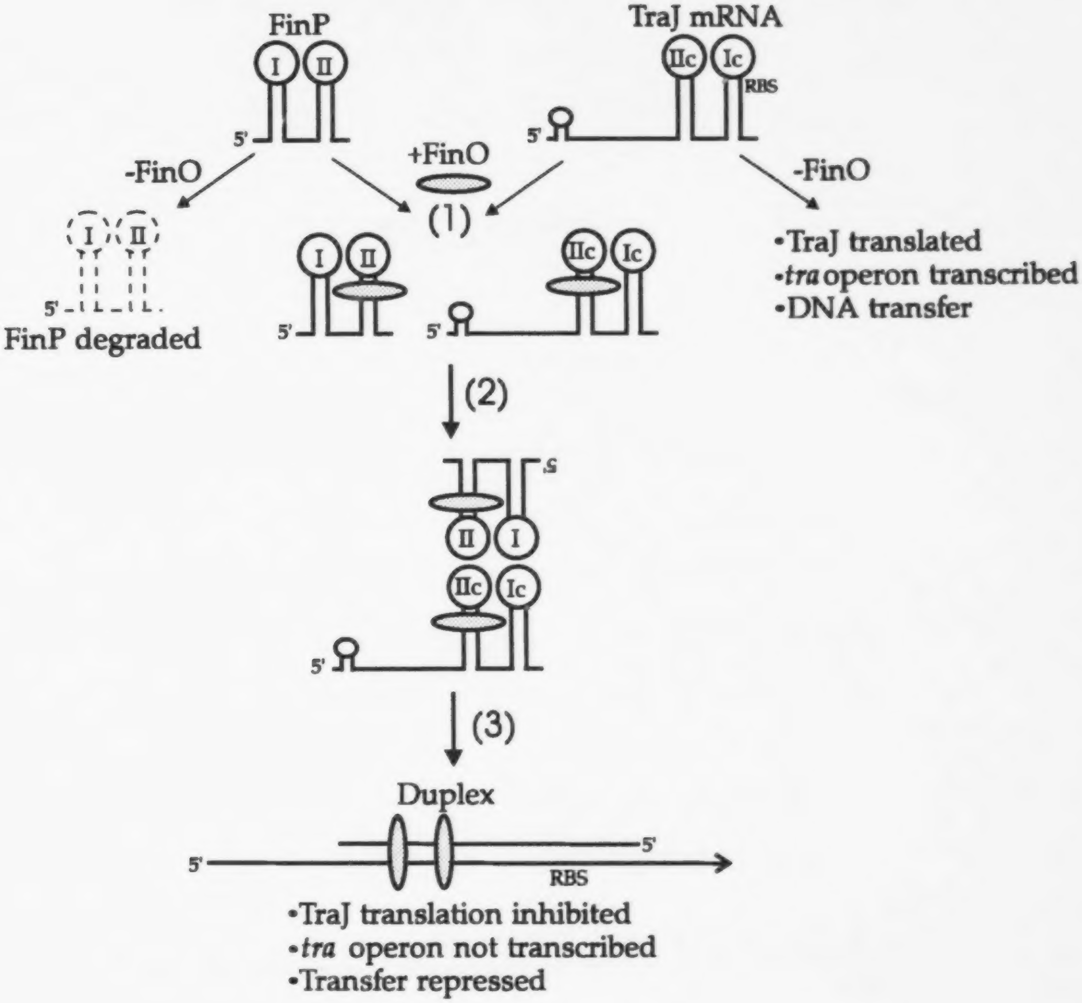
Tomizawa and Som (1984) have shown that inclusion of a large molar excess of Rom protein during RNA I/RNA II duplex formation causes a ~2-fold increase in the apparent second order rate constant (k_{app}). Similarly, analysis of the kinetics of FinP-TraJ211 duplex formation revealed that purified GST-FinO increased the k_{app} from 5×10^4 $M^{-1}s^{-1}$ to 2.4×10^5 $M^{-1}s^{-1}$, an increase of almost 5-fold. The mechanism by which FinO mediates this increase in k_{app} may indeed be very similar to Rom although FinO associates with both the antisense RNA and its target prior to formation of the duplex. Rom increases the rate of RNA I/RNA II duplex formation by reducing the rate of dissociation of the kissing complex (k_{-1}, see equation on p. 21). It is reasonable to propose that FinO has the same effect on the FinP-*traJ* mRNA kissing complex. In fact, it is equally likely that this complex, containing FinO, FinP, and *traJ* mRNA, is stable, and that the FinP-*traJ* mRNA duplex is not formed. This is supported by the observation that the complex is detectable only under non-denaturing conditions. Since the *traJ* RBS and its complement are partially located in the loop I domains, a simple kissing intermediate could conceivably inhibit TraJ translation by occluding the RBS.

Tomizawa (1984) and Persson *et al* (1990a) have demonstrated the requirement of a single-stranded region in stable duplex formation *in vitro* and *in vivo*. Although Rom does not have a significant effect on the rate of wild-type RNA I/RNA II pairing, it almost restores the rate of duplex formation when the 5'-terminal single-stranded region is removed from RNA I. Similarly, FinP has very short single-stranded tails (<6 nt), thus it is possible that either stable duplex formation does not occur *in vivo* or that FinO is required to increase the rate of duplex formation due to the lack of single-stranded regions in FinP RNA. We have not yet differentiated between these two possibilities.

Note that *in vivo*, FinO and FinP together cause a 50-100-fold decrease in the level of TraJ expression, suggesting that the observed 5-fold effect on the rate of duplex formation is not the sole function of FinO. Thus FinOP action may be a combination of FinP stabilization and the promotion of FinP-*traJ* mRNA duplex formation.

Based on the *in vitro* data reported here, the following model of FinOP-mediated inhibition of TraJ expression and, ultimately, fertility inhibition is proposed (Fig. 4h). FinP RNA is transcribed constitutively and, in the absence of FinO, is quickly degraded

Figure 4h: A model of FinOP function. The mechanism of FinOP-mediated inhibition of TraJ expression shown here is described in detail in the text. RBS = ribosome binding site. The loop domains of both RNAs are numbered I and II for FinP, and Ic and IIc (where c = complementary) for TraJ211. Drawing is not to scale.


FinP
I
II
5'
TraJ mRNA
IIc
Ic
RBS
5'
-FinO
+FinO
-FinO
(1)
I
II
5'
FinP degraded
I
II
5'
IIc
Ic
5'
•TraJ translated
•*tra* operon transcribed
•DNA transfer
(2)
5'
II
I
IIc
Ic
5'
(3)
Duplex
5'
5'
RBS
•TraJ translation inhibited
•*tra* operon not transcribed
•Transfer repressed

by an unknown ribonuclease. As a result, *traJ* mRNA is free to be bound by ribosomes and translated, resulting in the activation of *tra* operon transcription by the *traJ* gene product and the initiation of DNA transfer. However, if FinO is expressed in the cell, stem-loop II of FinP and its complement in *traJ* mRNA (step 1, Fig. 4h) are bound by FinO protein. The binding of FinO to FinP may prevent FinP degradation and thereby make it available for interaction with *traJ* mRNA. Duplex formation may be initiated via a transient intermediate similar to the kissing intermediates described earlier (step 2) (Persson *et al*, 1990a; Tomizawa, 1984). The formation of the stable duplex is assisted by binding of the FinO protein to both FinP and its target *traJ* mRNA (step 3), resulting in a structure which inhibits the translation of TraJ.

RNA-OUT of IS10 has been shown to regulate transposase expression by occluding the RNA-IN RBS (Ma and Simons, 1990). A similar mechanism is possible for FinP and *traJ* mRNA since their region of homology overlaps the *traJ* RBS. In contrast, cleavage of the stable CopA-CopT duplex by RNase III has been shown to be involved in the copy number control of plasmid R1 (Blomberg *et al*, 1990) although this is not the primary mechanism of regulation (Wagner *et al*, 1992). RNase III cleaves double-stranded RNA sequences non-specifically (Krinke and Wulff, 1990), therefore it is also possible that TraJ expression is inhibited due to FinP-dependent RNase III cleavage. Finally, FinO may be directly involved in the inhibition of TraJ expression, either by inhibiting TraJ translation or mediating destabilization of the *traJ* mRNA.

D. Materials and methods

I. Bacterial strains, plasmids, and media

pOX38-Km (Chandler and Galas, 1983) was the generous gift of K. Ippen-Ihler (Dept. of Medical Microbiology and Immunology, Texas A & M University) and was maintained in *E. coli* JC3272 (Achtman *et al*, 1971). pSnO104 (Lee *et al*, 1992) expressed the *finO* gene from plasmid R6-5. Cells were grown in TSB broth supplemented with 25 g ml^{-1} of the appropriate antibiotics.

II. Enzymes and chemicals

All restriction and modification enzymes were purchased from Boehringer Mannheim except for T7 RNA polymerase (Pharmacia) and Vent DNA polymerase (New England Biolabs). RNAguard RNase inhibitor was purchased from Pharmacia.

III. RNA isolation, Northern blotting

pOX38-Km transformed with either pSnO104 or pACYC184 (as a negative control) was grown to mid-log (OD$_{600}$ = 0.75). RNA was prepared from time samples using the hot phenol method (Frost *et al*, 1989).

For Northern blotting analysis, a 20 g aliquot of each RNA sample was electrophoresed on an 8% 8 M urea polyacrylamide gel at 450 V for 1 hour. The gel was electroblotted to Hybond-N nylon membrane (Amersham) using a Bio-Rad semi-dry blotting apparatus and UV-crosslinked in a Bio-Rad GS Gene Linker UV chamber. The FinP RNA was detected using a double-stranded ^{32}P-labeled PCR-generated fragment complementary to the *finP* gene. The blot was prehybridized for 1.5 hours at 37^{o}C in 2.5 x SSC, 5 x Denhardt's solution, 1.5 % SDS, 50% formamide, and 100 g ml^{-1} of *E. coli* strain W tRNA type XX (Sigma). The blot was hybridized for 6 hours before washing in 6 x SSC, 0.1% SDS at 37^{o}C for 15 min.

IV. *In vitro* transcriptions

FinP and TraJ211 were transcribed from PCR templates using T7 RNA polymerase as previously described (van Biesen *et al*, 1993). Whenever the term 'unlabeled' RNA is used in the text, the RNA has been transcribed in the presence of ^{3}H-UTP to allow for accurate determination of RNA concentration (Hjalt and Wagner, 1992).

Stem-loops I and II of FinP were similarly transcribed from PCR templates carrying synthetic T7 promoters. Stem-loop I consists of nucleotides 1 to 34 of FinP and stem-loop II contains nucleotides 35 to 78.

The fully formed ^{32}P-FinP/TraJ211 duplex was prepared by transcription of both RNAs together, boiling the completed reaction for 5 minutes, slowly cooling to room temperature and purifying from a 8% 8 M urea polyacrylamide gel.

V. GST and GST-FinO purification

A PCR-generated fragment containing the complete *finO* open reading frame was cloned into the BamH1 site of the pGEX-2T vector, immediately downstream of the thrombin cleavage site, to generate pGEX-FO2. GST and GST-FinO expression were induced from the *tac* promoter with 0.5 mM IPTG for 5 hours. Pelleted cells were stored at -20°C indefinitely prior to protein purification, without any detectable reduction in activity. Protein was purified using glutathione-agarose affinity (S-linked, Sigma) in 1.5% sarkosyl, 2% Triton X-100 (Frangioni and Neel, 1993). Protein bound to the beads was washed 5-6 times with 400 volumes TEB buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 g ml^{-1} BSA), and eluted using 1 volume of 20 mM reduced glutathione (Sigma) in TEB. Protein was stored at 4°C for no more than 24 hours prior to use to minimize loss of activity.

VI. RNA mobility shift assays

^{32}P-labeled RNA (10 000 CPM) was incubated with various concentrations of protein (GST or GST-FinO) in 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 g ml^{-1} BSA, 10% glycerol. Protein-RNA complexes were allowed to form at room temperature (~22^{o}C) for 30 min and aliquots were immediately loaded on a continuously running 8% non-denaturing polyacrylamide gel (100 V for 45 min) in 1 x TBE buffer (Sambrook *et al,* 1989). The gel was run for a only short distance to avoid extensive dissociation of the complex during electrophoresis. Competition assays to determine the specificity of GST-FinO binding were performed by combining the indicated amounts of unlabeled and labeled RNAs prior to incubation with the protein.

VII. Calculations

The association equilibrium constant (K_a) was calculated on the basis of the GST-FinO concentration which caused 50% of the labeled RNA to shift in the gel (Tsai *et al*, 1990). The calculated values were not corrected for the activity of non-specific protein-RNA interactions. Also, the values of K_a may be artificially low since we do not know what fraction of the GST-FinO preparations were active. K_a can be defined as K_a = $[BO]/([B]*[O_o])$, where [B] and [BO] are the concentrations of unbound FinO binding site and of FinO binding site bound by GST-FinO, and $[O_o]$ is the initial concentration of GST-FinO in the reaction. At 50% binding, [B] = [BO], thus K_a can be directly calculated as $K_a = 1/[O_o]$.

The relative dissociation constants of the two GST-FinO - duplex complexes were calculated as described by Tsai *et al* (1989). This calculation is based on the fractional maximum level of complex C1 ($fmax_{C1}$) detected in the experiment shown in Figure 4f: $Kd_{C1}/Kd_{C2} = ((fmax_{C1})^{-1}-1)^2$. Thus, when $fmax_{C1}$ is 0.4 (as shown in Results), the calculated ratio of Kd_{C1} to Kd_{C2} is 2.25.

VIII. *In vitro* FinP-TraJ211 binding assays

Binding assays were performed as described in Persson *et al* (1988) with the following modifications. In order to optimize both FinP-TraJ211 duplex formation and protein-RNA binding, the incubation buffer used was 30 mM Tris-Cl, pH 8.0, 5 mM $Mg(OAc)_2$, 0.5 mM EDTA, 50 mM NaCl, 50 g ml^{-1} BSA, plus GST (3.3×10^{-6} M) or GST-FinO (1.8×10^{-6} M).

All autoradiography was done with Kodak X-Omat AR film. Bands were quantitated by densitometry on a BioImage XRS 3cx scanner (Millipore).

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Chapter 5

Characterization of the *fisO305* mutation of FinP antisense RNA

A. Introduction

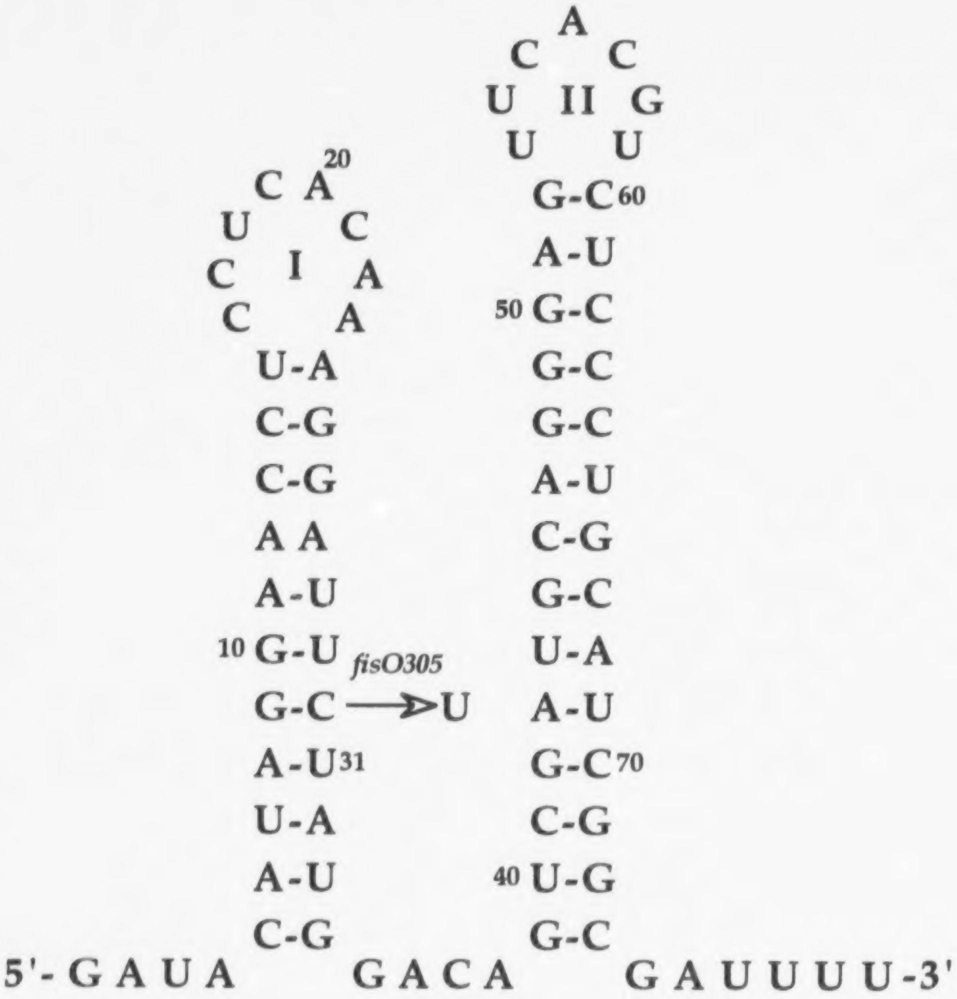
Naturally occurring antisense RNA regulatory systems have been identified in a wide variety of prokaryotic and eukaryotic organisms (for a review, see Simons, 1993). Among the prokaryotes, these antisense RNAs are most often associated with mobile elements such as bacteriophages, plasmids and transposons (Nordström *et al*, 1993). Antisense RNAs are small (~100 nt) molecules which tend to exhibit a highly ordered secondary structure and inhibit gene expression at the post-transcriptional level. Generally, a stable duplex is formed between the antisense RNA and its target mRNA, resulting in negative regulatory effects such as mRNA degradation (Blomberg *et al*, 1990) or the inhibition of mRNA translation (Kittle *et al*, 1989).

The mechanism of antisense RNA mediated inhibition of gene expression has been thoroughly examined in a number of systems (Nordström *et al*, 1993). In each case, the secondary structure of the RNAs are crucial to their function. For example, the stem domain of RNA-OUT of IS10 is required for the maintenance of its stability *in vivo* (Case *et al*, 1989), and the single-stranded regions of RNA I of plasmid ColE1 (Tomizawa, 1984) and CopA of plasmid R1 (Persson *et al*, 1990) have been shown to be necessary for efficient antisense RNA-target RNA duplex formation. Similarly, the stem domain of the FinP antisense RNA of F-like conjugative plasmids appears to play a role in the formation of a ribonucleoprotein complex with the FinO protein, resulting in the inhibition of TraJ expression (van Biesen and Frost, 1994).

This paper reports the partial characterization of a mutation in FinP antisense RNA, *fisO305*, which has been predicted to affect the site of interaction between the FinP RNA and the FinO protein (Finnegan and Willetts, 1971, 1973; Frost *et al*, 1989). FinP and FinO mediate the fertility inhibition of F-like conjugative plasmids, causing a 0-1000-fold decrease in the rate of conjugative transfer of DNA. Together, they inhibit expression of the TraJ protein which acts as a transcriptional activator of the transfer (*tra*) operon. Recently, FinO has been found to bind directly to both FinP and *traJ* mRNA, resulting in both an increase in the steady-state levels of FinP *in vivo* and an increase in the rate of FinP-*traJ* mRNA duplex formation *in vitro* (van Biesen and Frost, 1994). The stable duplex is thought to prevent TraJ translation due to occlusion of the ribosome binding site.

Mutational analysis of the *finP* gene reported by Finnegan and Willetts (1971, 1973) revealed two types of *finP* mutants, only one of which was fully complementable *in trans*. The second class of mutants, which were poorly complemented, were postulated to affect the site of FinP-FinO interaction and thus called *fisO* mutants. FinP RNAs carrying the *fisO305* mutation are called FisP. Frost *et al* (1989) demonstrated that *finO* expressed *in trans* caused an increase in the steady-state levels of wild-type FinP *in vivo*. However, this effect was not observed with FisP RNA, suggesting that the site of FinO action had been altered. The *fisO305* (C30→U30) mutation is located in stem-loop I of FinP (Fig. 5a) and causes a predicted decrease in the free energy of the stem domain. However, a second-site mutant of FisP (G9→A9) which restored the free energy of this structure was found to exhibit the same phenotype, suggesting that the effect of the *fisO305* mutation was not due to destabilization of the stem domain (Frost *et al*, 1989). Structure-disrupting mutations in both stems I and II were found to have a milder effect on transfer frequencies and did not show a significant decrease in the steady-state levels of FinP RNA. In a similar analysis of the *finP* gene of plasmid R1, Koraimann *et al* (1991) demonstrated that mutations which caused minor structural alterations in stem domain II did not have a drastic effect on transfer frequencies and thus did not exhibit a *fisO* phenotype.

Figure 5a: The *fisO305* mutation of FisP RNA. A graphical representation of the FinP RNA is shown. The location and nature of the *fisO305* mutation is shown at position 30.



20
C A
U C
C I A
C A
U-A
C-G
C-G
A A
A-U
10 G-U
fisO305
G-C → U
A-U 31
U-A
A-U
C-G
A
C C
U II G
U U
G-C 60
A-U
50 G-C
G-C
G-C
A-U
C-G
G-C
U-A
A-U
G-C 70
C-G
40 U-G
G-C
5'-G A U A
G A C A
G A U U U U-3'

Here we report the phenotype of the FisP mutant both *in vivo* and *in vitro*. Contrary to previously reported results, the location of the *fisO305* mutation in stem-loop I of FinP does not appear to be a part of the FinO binding site. Also, FisP appears to form a complex with *traJ* mRNA with an apparent second order rate constant of duplex formation similar to that for wild-type FinP. However, FinO appears to be unable to protect the FisP RNA from RNase degradation *in vivo*, and together, FinO and FisP cause only a marginal decrease in the steady-state level of *traJ* mRNA.

B. Results

I. FinO protein binds to FisP RNA *in vitro*

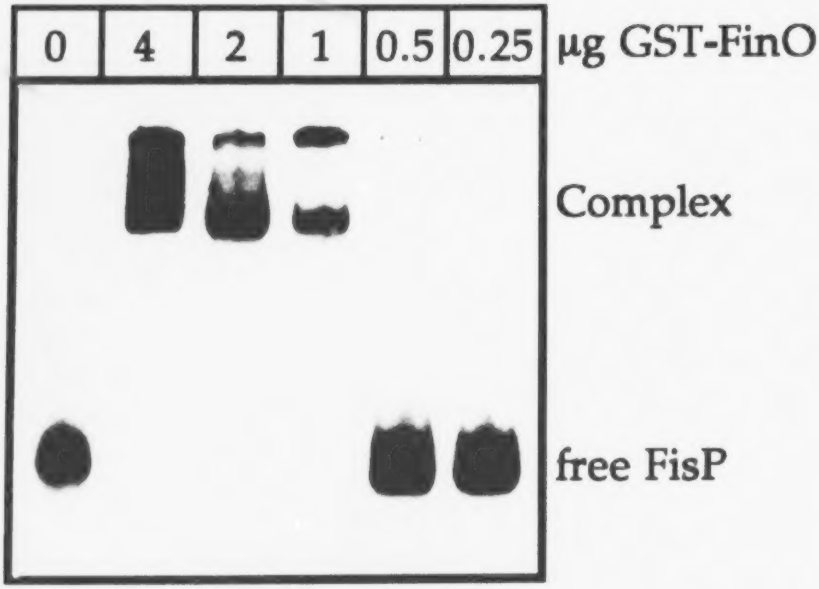
The ability of FinO protein to bind directly to the FinP antisense RNA has previously been demonstrated (van Biesen and Frost, 1994; Chapter 4). FisP RNA was synthesized *in vitro* from DNA templates generated by PCR using plasmid ED1844 as a template. ED1844 is a mutant derivative of F carrying the *fisO305* mutation. FinO was purified as a fusion with glutathione-S-transferase (GST-FinO) and retains wild-type FinO activity both *in vivo* and *in vitro* (van Biesen and Frost, 1994; Chapter 4).

Using mobility shift assays, we examined the ability of GST-FinO to retard the mobility of the FisP RNA (Fig. 5b). Co-incubation of a molar excess of GST-FinO with ^{32}P-FisP caused a marked reduction in the mobility of the FisP band in the gel. The dependence of FisP mobility on GST-FinO concentration was used to determine the association equilibrium constant (K_a) of the complex. The calculated value of K_a, $\sim 4 \times 10^5$ M^{-1}, is similar to that determined for the binding of FinO to wild-type FinP RNA (van Biesen and Frost, 1994; Chapter 4). The specificity of FinO-FisP binding was demonstrated by the observation that the formation of the complex was severely inhibited by the addition of unlabeled FisP, whereas binding was relatively unaffected in the presence of a large excess of tRNA (data not shown).

II. FisP forms a duplex with *traJ* mRNA *in vitro*

The kinetics of FinP - *traJ* mRNA duplex formation have been shown to be comparable to other naturally occurring antisense RNA systems, with an apparent second order rate constant of duplex formation (k_{app}) of 5×10^5 $M^{-1}s^{-1}$ (van Biesen *et al*, 1993; Chapter 3). This type of interaction is thought to be affected by changes in the secondary structure of the RNA molecules, an effect which has been demonstrated for a number of antisense RNAs (Eguchi and Tomizawa, 1991; Persson *et al*, 1990).

Figure 5b: Mobility shift assay of FisP RNA by GST-FinO. Gel-purified FisP RNA was co-incubated with various amounts of GST-FinO. The locations of the free FisP and the FinO-FisP complex are shown. The reaction and electrophoresis conditions were identical to those described in figure 4d.


0
4
2
1
0.5
0.25
µg GST-FinO
Complex
free FisP

Since the *fisO305* mutation causes a decrease in the free energy of the stem I domain of FinP, and perhaps an alteration in the secondary structure, we postulated that this RNA would exhibit a decreased ability to form a duplex with a *traJ* transcript carrying the complementary mutation.

FisP and TraS211, a 211 nt truncated *traJ* transcript carrying the complementary *fisO305* mutation, were synthesized *in vitro* from DNA templates synthesized by PCR. These PCR-generated templates were synthesized from a *fisO305* derivative of the F plasmid, ED1844. Also, a truncated wild-type *traJ* mRNA, TraJ211 (van Biesen *et al*, 1993) was used in a separate duplex formation assay.

^{32}P-FisP was incubated in the presence of a molar excess of unlabeled TraS211 or TraJ211 and samples were withdrawn at the times indicated (Fig. 5c.i). The kinetics of FisP-TraJ211 duplex formation are represented graphically in Fig. 5c.ii and were used to calculate the apparent second order rate constant of duplex formation. Although FisP does not mediate fertility inhibition *in vivo*, it is able to form a duplex with *traJ* mRNA at rates comparable to those observed for wild-type FinP ($k_{app} = 5 \times 10^5$ $M^{-1}s^{-1}$), regardless of whether the target RNA carries the complementary mutation. This surprising result indicated that the formation of the FinP-TraS211 duplex was unaffected by changes in the sequence of the stem-loop I domains of both RNAs. Also, a stable duplex was able to form between the FisP and the wild-type TraJ211, suggesting that a mismatch between the molecules in this region was not inhibitory to duplex formation.

III. FisP has a decreased effect on the steady-state levels of *traJ* mRNA *in vivo*

van Biesen *et al* (1993) have demonstrated that the FinOP complex causes a 50-fold reduction in the intracellular concentration of *traJ* mRNA *in vivo*. This is thought to be due to a decrease in *traJ* mRNA stability as a result of duplex formation with FinP.

Figure 5c: The kinetics of FisP-TraJ211 duplex formation.

i. The kinetics of FisP-TraJ211 duplex formation. ^{32}P-labeled FisP RNA (~5 x 10^{-10} M) was coincubated with 5 x 10^{-9} M unlabeled TraJ211 RNA. Samples were taken at the indicated time points and diluted in 2x formamide loading buffer prior to loading on an 8% non-denaturing polyacrylamide gel.

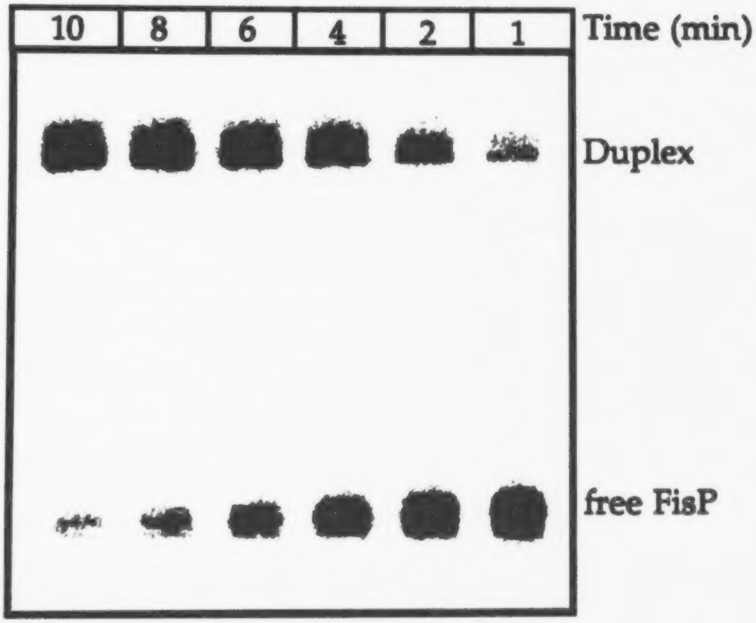
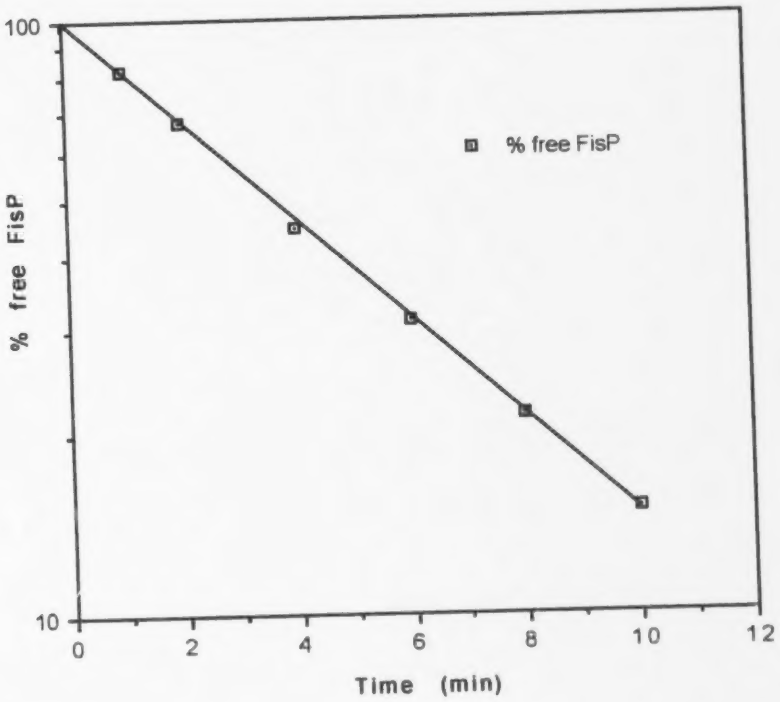
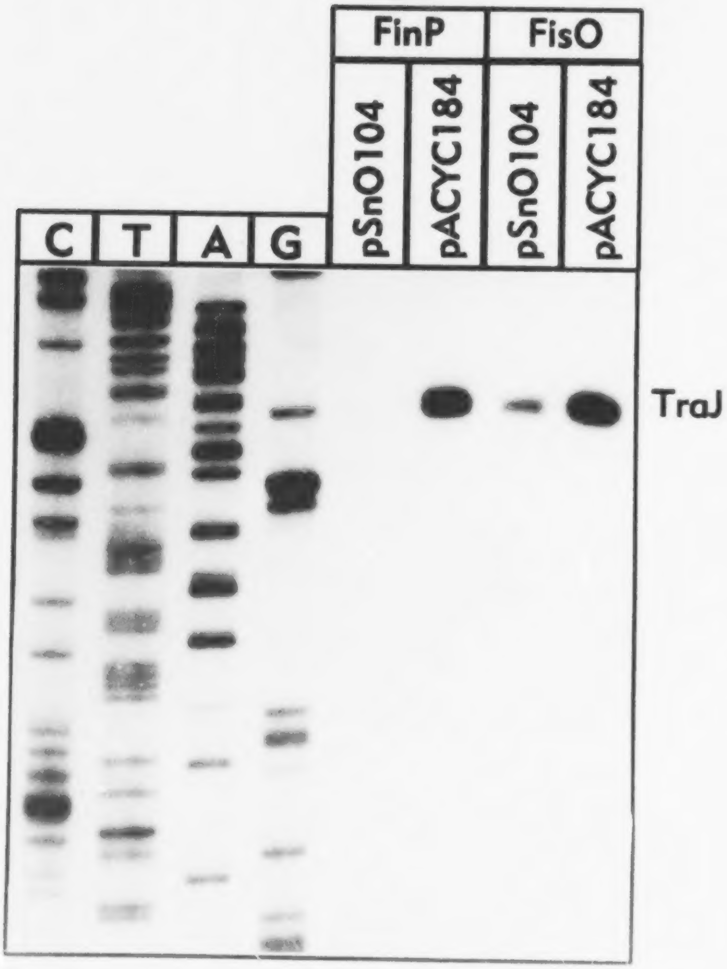

10
8
6
4
2
1
Time (min)
Duplex
free FisP

Figure 5c: ii. Graphical representation of the experiment in panel 5c.i. The ratio of uncomplexed FisP to RNA duplex was measured using a Molecular Dynamics Phosphorimager. The amount of free FinP, as a percent of the total FinP in the reaction, is depicted as a function of time.


100
10
% free FisP
0
2
4
6
8
10
12
Time (min)
% free FisP

The RNA extracted from the strains carrying ED1844 (FisO) with and without FinO was also used for the primer extension analysis of the *traJ* mRNA. In the absence of FinO (lane FisO:pACYC184, Fig. 5d) the full-length *traJ* mRNA was readily detectable. The co-expression of FinO with FisP (lane FisO:pSnO104, Fig. 5d) caused only a 3-fold decrease in the intracellular levels of *traJ* mRNA, as compared to the 50-fold decrease caused by the wild-type FinP RNA, suggesting that the FisP-FinO complex is unable to mediate the degradation of the *traJ* mRNA *in vivo*.

Figure 5d: The effect of FisP and FinO on *traJ* mRNA *in vivo*. Primer extension analysis of *traJ* mRNA from pOX38/Km and ED1844 (*fisO305*). RNA was analyzed from strains with (+pSnO104) and without (+pACYC184) *finO*. Dideoxy sequencing reactions were carried out on plasmid DNA (pSJ99) using the same primer. The full-length *traJ* transcript is labeled.


FinP
FisO
C
T
A
G
pSnO104
pACYC184
pSnO104
pACYC184
TraJ

C. Discussion

Mutational analysis of the FinP gene by Finnegan and Willetts (1971) indicated that there was one class of mutants, termed *fisO*, which was thought to represent the target of the product of the *finO* gene. This idea was further reinforced by the observation that *finO* expressed *in trans* did not affect the steady-state levels of FisP RNA (FinP carrying the *fisO305* mutation), although the intracellular levels of wild-type FinP were significantly increased (Frost *et al*, 1989). The two *fisO* mutants isolated by Finnegan and Willetts (1971) were later found to be identical in DNA sequence (Frost *et al*, 1989), suggesting that the site of FinO-FinP interaction was a limited region of the stem I domain of FinP.

Previously, we have demonstrated by electrophoretic mobility shift assays that the FinO protein binds directly to the stem-loop II domain of FinP, rather than stem-loop I (van Biesen and Frost, 1994). Here we have shown that the GST-FinO protein bound to the FisP RNA with the same affinity as the wild-type FinP RNA, despite the presence of a base substitution in the stem-loop I domain. The observation that alteration of the sequence or free energy of stem-loop I did not affect FinP-FinO binding supports the conclusion that FinO binds to the stem-loop II domain of FinP.

Many naturally occurring antisense RNAs adopt a highly ordered secondary structure which is thought to be important for both RNA stability and also rapid formation of the antisense RNA -target RNA complex. Thus, an increase in the free energy of the stem I domain of the FinP RNA was expected to reduce the apparent second order rate constant of duplex formation (k_{app}). The duplex formation experiments were performed using purified FisP RNA combined with either wild-type TraJ211 RNA or the mutant TraS211 RNA, which carried the mutation complementary to the *fisO305* mutation of FisP. The FisP-TraS211 complex was predicted to be a continuous RNA duplex, whereas the FisP-TraJ211 complex would contain a single mismatched base pair. Surprisingly, the calculated value of k_{app} for FisP duplex formation with either of the two TraJ targets was identical to that for wild-type FinP. These results suggest that the alteration of the sequence of stem-loop I does not have an effect on the kinetics of duplex formation, nor does the presence of a mismatch between the antisense RNA and its target in the stem-loop I region. Persson *et al* (1990) have shown that only one of the two stem-loop domains of CopA antisense RNA is important for efficient duplex formation. It is possible that a similar situation exists for FinP, where the stem-loop II domain may be more important for FinO binding and efficient duplex formation. This proposal is supported by the observation that mutations on the loop II domain of R1 FinP have a more deleterious effect than those in loop I, suggesting that the specificity of the FinP-*traJ* mRNA interaction lies in the loop II region (Koraimann *et al*, 1991).

Although the alteration of stem-loop I of FinP did not affect the kinetics of duplex formation, the *in vivo* stability of FisP has been shown to be significantly decreased. Previous results have indicated that the steady-state levels of FisP RNA *in vivo* were markedly reduced compared to the wild-type FinP (Frost *et al*, 1989). Initially, these results were interpreted to suggest that the *fisO305* mutation altered the FinO binding site, thereby inhibiting its ability to protect the RNA from cleavage. However, it is equally possible that this alteration of the stem-loop I structure optimizes a pre-existing RNase cleavage site, thereby increasing the rate of FisP degradation and negating the inhibitory effect of the bound FinO. Similarly, disruptions of the secondary structure of the stem domain of RNA-OUT of IS10 have been shown to cause a dramatic decrease in the *in vivo* half-life of this RNA (Case *et al*, 1989).

The co-expression of FinP and FinO has been shown to reduce the steady-state levels of *traJ* mRNA by approximately 50-fold (van Biesen *et al*, 1993). This effect may be due to FinP-mediated destabilization of the *traJ* mRNA, perhaps as the result of the formation of a stable RNA duplex which acts as an efficient substrate for cleavage by RNase III. Although FisP has been shown to form a duplex efficiently with *traJ* mRNA *in vitro*, it does not have a significant effect on the steady-state levels of *traJ* mRNA *in* *vivo.* Primer extension analysis of the *traJ* mRNA revealed that the presence of FisP and FinO caused a less significant decrease in the intracellular levels of *traJ* mRNA. We propose that FisP RNA is fully capable of inhibiting TraJ synthesis and also destabilizing the *traJ* mRNA *in vivo.* However, the effect of FisP is abrogated by its increased rate of degradation, thereby reducing the effective concentration of this inhibitor of TraJ translation. Note that a second site mutation of FisP (G9→A9) does not restore FinP activity, although it is predicted to decrease the free energy of the stem domain. We predict that the replacement of the wild-type G-C base pair by an A-U base pair does not decrease the enhanced rate of FisP degradation. Further mutational analysis of both stem domains is required to accurately identify the minimal structures required for efficient duplex formation, FinO binding, and RNA stability.

Characterization of the FisP mutant has served to identify the importance of the stem-loop II region of the FinP antisense RNA. Interestingly, the *traJ* mRNA RBS is located in the region complementary to stem-loop I suggesting that this structure may be required to mediate inhibition of TraJ translation but not the formation of the inhibitory duplex. However, the mechanism of FinP-mediated inhibition of TraJ translation has not yet been demonstrated to be due to either occlusion of the TraJ RBS or destabilization of the *traJ* mRNA. Indeed, RNase III - mediated cleavage of the CopA-CopT duplex was shown to affect expression of RepA *in vivo* (Blomberg *et al*, 1990), although the primary mode of translation inhibition was later shown to be the occlusion of the RBS of a translationally coupled upstream ORF (Wagner *et al*, 1992). We are presently attempting to identify the nuclease which mediates cleavage of the FinP RNA *in vivo* in order to characterize its role in the regulation of TraJ expression.

D. Materials and Methods

I. Bacterial strains, plasmids and media

pOX38/Km (Chandler and Galas, 1983) was the generous gift og K. Ippen-Ihler (Dept. of Medical Microbiology and Immunology, Texas A&M University) and was maintained in *E. coli* JC3272 (Achtman *et al*, 1971). ED1844 was the generous gift of David Finnegan (Edinburgh) and was maintained in *Escherichia coli* JC3272 (Achtman *et al*, 1971). *finO* (from plasmid R6-5) was provided *in trans* by plasmid pSnO104 (Lee *et al*, 1992). The DNA sequences shown in figure 5e was generated using plasmids pSJ99 (Lee *et al*, 1992) as template. Cells were grown in tryptic soy broth supplemented with the appropriate antibiotics at 25 μg ml^{-1}.

II. Enzymes and chemicals

All restriction and modification enzymes were purchased from Boehringer Mannheim except for T7 RNA polymerase (Pharmacia) and Vent DNA polymerase (New England Biolabs). RNAguard RNase inhibitor was purchased from Pharmacia.

III. *In vitro* transcriptions

DNA for *in vitro* transcription of FisP and TraS211 were generated by PCR using plasmid ED1844 as a template, as previously described for the wild-type FinP and TraJ211 RNAs (van Biesen *et al*, 1993). *In vitro* transcriptions were performed as previously described (van Biesen *et al*, 1993). Whenever the term 'unlabeled RNA' is used in the text, the RNA has been transcribed in the presence of ^{3}H-UTP to allow for accurate determination of RNA concentration (Hjalt and Wagner, 1992).

IV.GST-FinO purification, mobility shift assays and FinP-TraJ211 duplex reactions

The purification of the GST-FinO fusion protein and the electrophoretic mobility shift assays were performed as previously described (van Biesen and Frost, 1994). The kinetics of FinP(FisP) - TraJ211(TraS211) duplex formation were performed as previously described (van Biesen *et al*, 1993).

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Chapter 6

General Discussion and Conclusions

A. General Discussion

The detailed analysis of several naturally occurring antisense RNA systems has revealed that each antisense RNA - target RNA pair forms an inhibitory complex in a similar manner (Nordström *et al*, 1993; Simons, 1993). However, each system also exhibits unique characteristics such as differences in secondary structure, mechanisms of target binding and even association with protein catalysts. The analysis of the FinP antisense RNA of the F plasmid was undertaken to identify both the common features it shares with other antisense RNA systems and also those attributes which cause it to behave in a unique manner.

The FinOP fertility inhibition system of F-like conjugative plasmids exhibits a variety of unusual features which differentiate it from previously characterized antisense RNA systems. The most unusual feature of this system is that its function is entirely dependent on the presence of a third component, the product of the *finO* gene. The analysis of the FinOP system presented in this thesis has elucidated a role for FinO in mediating the inhibition of TraJ expression.

The characterization of *finO* was initiated with the cloning of the ColB2 *finO* gene which exhibits a lower level of fertility inhibition than previously cloned *finO* genes (Willetts and Maule, 1986). Surprisingly, the sequence of the ColB2 *finO* gene was almost identical to those sequences previously reported for plasmids R100 and R6-5. However, the presence of an additional ORF, *orf286*, upstream of *finO* in some plasmids caused a significant increase in the activity of FinO. Over-expression studies and Northern analysis of the transcripts revealed that this ORF, when co-transcribed with *finO*, increased the stability of the *finO* mRNA *in vivo*, thereby increasing the level of FinO expression. The computer-predicted secondary structure of the *orf286-finO* transcript suggested that *orf286* is able to stabilize the *finO* mRNA via an extended region of sequence complementarity, resulting in the formation of a paired region of

RNA that may be resistant to RNase-mediated degradation. This study revealed that there is only a single allele of *finO* and that the level of its expression is dependent upon the context in which it is transcribed. Also, the efficiency of the fertility inhibition system was shown to be directly dependent on the intracellular concentration of *finO* mRNA.

In order to characterize the function of the FinO protein, it was first necessary to study the nature of the formation of the FinP-*traJ* mRNA duplex. Computer predictions, mutational analysis (Koraimann *et al*, 1991) and phylogenetic analysis (Finlay *et al*, 1986) have been used to predict the secondary structure of FinP RNA. Using structure-specific RNases, the structure of FinP was confirmed to consist of two stem-loop domains, each of which is flanked by short single-stranded regions. The target of the FinP RNA, the 5' untranslated region of *traJ* mRNA, was found to fold into a structure complementary to that of FinP. The *traJ* mRNA RBS is located near the top of stem-loop I, suggesting that this may be a target for the inhibition of TraJ translation by FinP. These highly ordered structures are characteristic of many naturally occurring antisense RNAs and are thought to contribute to the stability of the RNA and the ability to form an inhibitory duplex.

Analysis of the kinetics of the formation of the FinP-*traJ* mRNA duplex *in vitro* revealed that these two RNAs formed a complex at a rate comparable to previously characterized systems ($k_{app}=5 \times 10^5$ $M^{-1}s^{-1}$). These studies were performed in the absence of the FinO protein, indicating that FinP has the ability to form a duplex with *traJ* mRNA *in vitro*, although FinO is absolutely required for FinP function *in vivo*. It is likely that the FinP-*traJ* mRNA duplex is formed via a series of transient intermediates similar to those proposed by Tomizawa (1984,1990) and Persson *et al* (1990a,b), including the formation of a kissing complex mediated by the loop domains of the two RNAs. The formation of a kissing complex between FinP and *traJ* mRNA is further evidenced by the observation that mutations in either loop domain decrease the activity of FinP (Koraimann *et al*, 1991). However, it is interesting to note that the k_{app} value for FinP-*traJ* mRNA duplex formation was high, since FinP lacks extended single-stranded regions of RNA. The importance of single-stranded regions has been demonstrated for both the RNA I antisense RNA of plasmid ColE1 and also the CopA antisense RNA of plasmid R1.

The fate of FinP and *traJ* mRNA *in vivo* was studied by Northern blotting and primer extension analysis. FinP RNA, when expressed in the absence of FinO, was present at undetectable concentrations in the cell. Interestingly, full-length FinP could be detected when it was co-expressed with the FinO protein, suggesting that FinO either inhibits the activity of a FinP-specific RNase or it binds directly to FinP in order to prevent its cleavage. Thus, the presence of FinO increases the effective concentration of active FinP in the cell, thereby increasing the efficiency of the inhibition of TraJ expression. The FisP mutant of FinP, which carries the *fisO305* mutation (C30→U30) in stem-loop I, was predicted to be unable to interact with the FinO protein based on genetic and biochemical analyses (Finnegan and Willetts, 1971; Frost *et al*, 1989). The observation that FinO was unable to protect FisP from degradation suggested that the *fisO305* mutation alters either the binding or the inhibitory activity of FinO (Frost *et al*, 1989). However, GST-FinO was shown to bind to FisP with a wild-type affinity, indicating that the *fisO305* mutation does not affect the FinO binding site.

FinO expression also affects the levels of *traJ* mRNA in the cell, although it does so in an indirect manner. In the absence of FinO, full length *traJ* mRNA is readily detectable by primer extension, even in the presence of FinP. However, the co-expression of FinO and FinP caused a significant reduction in the steady-state levels of *traJ* mRNA. This effect is likely a result of the increase in FinP activity resulting from FinO expression. Analysis of the detectable decay products of *traJ* mRNA suggest that the formation of the FinP-*traJ* mRNA complex *in vivo* results in the cleavage of the duplex by RNase III. This destabilization of the *traJ* transcript by FinP and FinO may be an important mechanism of TraJ regulation, however it is equally likely that occlusion of the *traJ* RBS by FinP is the primary cause of the inhibition of TraJ translation. Similarly, the CopA-CopT duplex is processed *in vivo* and *in vitro* by RNase III (Blomberg *et al*, 1990), but its primary mode of inhibition is the occlusion of the RBS of a translationally coupled upstream ORF called *tap* (Wagner *et al*, 1992). The co-expression of FinO with the *fisO305* mutant of FinP caused only a 3-fold decrease in the detectable level of *traJ* mRNA, an effect which may be a result of its demonstrated instability or an inability to form an inhibitory complex with *traJ* mRNA. Note that the cleavage of *traJ* mRNA by RNase III in the absence of FinP, or before duplex formation, has not been ruled out.

I. The specificity of FinO binding

Sequence-specific double-stranded RNA binding proteins are virtually unknown since the functional groups of the bases are buried in the deep and narrow major groove of the A-form helix (Mattaj, 1993). The accessible minor groove does not exhibit sequence specific characteristics and thus protein interactions in this region tend to be non-specific. Generally, RNA binding proteins make sequence-specific contacts at structural disruptions of the RNA helix, such as loops, bulges or mismatched base pairs. These structures allow the functional groups of the protein to recognize the salient features of the bases in the helix. The Rom protein is thought to recognize stem-loop I of RNA I via a bulged out C residue near the top of the stem (Moser *et al*, 1984). How then, does the FinO protein recognize its binding site? The data presented in this thesis do not explain the mechanism of this unusual interaction, however it is likely that FinO recognizes extended regions of double-stranded RNA in a sequence- or structure-specific manner.

The *in vitro* analysis of the function of the FinO protein was facilitated by the purification of FinO as a fusion with glutathione-S-transferase. Assuming that the *in vivo* steady-state levels of GST-FinO and wild-type FinO are comparable, the GST-FinO fusion retained wild-type levels of FinO activity *in vivo*, indicating that the presence of the GST moiety did not significantly affect FinO activity. *In vitro*, GST-FinO bound specifically to the second stem-loop domain of FinP RNA. This highly unusual interaction is predicted to be structure-specific since FinO also bound to the complementary structure in *traJ* mRNA and to at least two positions on the stable FinP-*traJ* mRNA duplex.

The interaction of FinO with *traJ* mRNA and the FinP-*traJ* mRNA duplex suggests that this protein has a role *in vivo* in addition to its ability to stabilize FinP RNA. In addition, although the FisP mutant of FinP is not stabilized by FinO *in vivo*, it exhibited the same affinity for FinO *in vitro* as did the wild-type FinP. In retrospect, this is not surprising since the FinO binding site was shown to be located on the second stem-loop domain of FinP, whereas the *fisO305* mutation of FisP is located in stem domain I. This result suggests that the FisP RNA is unstable *in vivo* for reasons other than a lack of interaction with FinO, but perhaps is due to the creation of an RNase cleavage site as a result of the mutation.

What, then, are the specific determinants for the binding of FinO to its binding site? It is possible that the interaction is dependent only on the presence of an extended region of double-stranded RNA. GST-FinO bound to a duplex which consisted only of the stem-loop I domains of FinP and *traJ* mRNA. Also, the FinP-*traJ* mRNA duplex appeared to be bound by as many as 3-4 molecules of FinO, suggesting that the mechanism of binding is not sequence-specific. However, the idea that FinO interacts non-specifically with double-stranded RNA is unlikely, since as much as 40% of the total cellular RNA is double-stranded. The steady-state levels of FinO protein are very low, suggesting that its binding to FinP and *traJ* mRNA must be specific in order for the protein to exert its effect. The observation that the level of fertility inhibition was a function of the intracellular concentration of FinO indicates that this protein is a limiting factor in the regulation of TraJ expression.

There are several other observations which argue that the binding of FinO to its target is specific. First, GST-FinO bound only to the stem-loop II domain of FinP, and not to stem-loop I. In addition, a mutant of FinP (*finP302*), which introduces a C-A mismatch into the stem II domain, was stabilized *in vivo* by the presence of FinO (Frost *et al*, 1989). This indicated that FinO binding was unaffected by a disruption near the base of the stem which allowed the formation of only 10 paired bases, and that the determinants required for FinO binding were not altered to a significant degree. Similarly, *traJ* mRNA from plasmid R100 contains a C-A mismatch in the middle of stem II and a corresponding G-U base pair in FinP (Finlay *et al*, 1986), but is still subject to the control of the FinOP system. Thus, if the interaction of FinO with its binding site requires an uninterrupted region of double-stranded RNA, then it is reasonable to suggest that the binding of FinO to FinP may be more important than its binding to *traJ* mRNA.

Based on the observations presented above, it is likely that the interaction between FinO and FinP/*traJ* mRNA may have an element of specificity. It is unlikely that FinO makes sequence-specific contacts with the double-stranded stem-loop II domain, because FinO acts on all of the alleles of FinP, which differ mainly in their loop sequences. Thus, it may recognize a sequence-induced conformational change in the structure of the RNA. A cursory examination of the sequence of the stem II domain reveals that there are five GC base pairs repeated at approximately 4 bp intervals, whereas only two such base pairs appear in the stem I domain. However, when a stable duplex is formed between the stem-loop I domains of FinP and *traJ* mRNA, a similar pattern of 4 GC bp repeats is created, perhaps explaining the ability of GST-FinO to bind to this molecule. It is interesting to note that these GC base pairs are oriented such that all of the G residues appear on one strand and the corresponding C residues on the other strand. Further experimental analysis is required to confirm this model.

Mattaj (1993) has recently introduced nine classes of RNA binding proteins. Several of these groups are characterized by specific RNA recognition motifs such as zinc fingers, basic/aromatic islands, or arginine-rich subsequences. Examination of the amino acid sequence of the FinO protein does not reveal the presence of any of these consensus sequences. Furthermore, FinO does not appear to have a significant level of homology to any other known proteins. Nonetheless, its ability to bind to double-stranded RNA molecules indicates that it should be included in the "double-stranded RNA binding motif" class of proteins (Mattaj, 1993).

II. The effect of FinO on FinP-*traJ* mRNA duplex formation

The observation that FinO binds to both FinP and *traJ* mRNA prompted the analysis of the kinetics of FinP-*traJ* mRNA duplex formation in the presence and absence of FinO. As described above, FinP forms a duplex with *traJ* mRNA at a rate comparable to previously characterized systems. However, the addition of GST-FinO to the reaction increases the apparent second order rate constant of duplex formation by a further 5-fold, suggesting that FinO promotes FinP-*traJ* mRNA duplex formation. Although the FisP mutant has been shown to be capable of forming a duplex with *traJ* mRNA *in vitro*, it is not known whether this interaction is catalyzed by FinO.

The effect of FinO is comparable to that observed for the Rom protein of plasmid ColE1. Rom has been shown to increase the rate of RNA I/RNA II duplex formation by 2-fold *in vitro*. This activity results in a corresponding 2-fold decrease in the plasmid copy number *in vivo*. Rom has been shown to catalyze duplex formation by inhibiting dissociation of the kissing complex, thereby favoring the formation of the stable duplex. FinO may act in a similar fashion by preventing the dissociation of FinP from *traJ* mRNA. In fact, it is possible that a complete and stable duplex between FinP and *traJ* mRNA does not form *in vivo*, and that translation of TraJ is inhibited by an intermediate complex containing FinO, FinP and *traJ* mRNA. Unlike FinO, Rom is unable to bind to either the antisense RNA, the target RNA, or the stable duplex and the interaction with the RNAs occurs only transiently during duplex formation. Although the increase in the rate of FinP-*traJ* mRNA duplex formation by FinO *in vitro* is only 5-fold, its effect *in vivo* is as much as 1000-fold, indicating that the function of FinO is more complex than that of Rom.

III. What is the role of FinO in regulating bacterial conjugation?

It is apparent that the FinO protein is absolutely required to mediate the inhibitory activity of FinP *in vivo*. However, FinP and *traJ* mRNA rapidly form a duplex *in vitro* without the assistance of FinO. Although FinP is unstable *in vivo* in the absence of FinO, it can be stabilized by introducing mutations in the stem (for example, see the *finP300B* mutation in Frost *et al*, 1989) which decrease the free energy of the molecule or alter a nuclease cleavage site. Why then does *E. coli* encode a protein simply to stabilize FinP RNA and to mediate a small increase in the rate of duplex formation?

An analysis of the events which occur *after* the conjugative event may provide the answer to this question. First, the activity of the fertility inhibition system reduces the rate of transfer of conjugative plasmids by up to 1000-fold. With such a low level of transfer, how does the plasmid propagate itself through a population of bacterial cells? After the initial transfer of the plasmid to a new recipient, the *tra* operon is temporarily derepressed, thereby allowing a high frequency of transfer (HFT) to occur. This short-lived HFT or "transient derepression" event allows the plasmid to be distributed throughout the population, until repression is re-established by the FinOP system (Lundquist and Levin, 1986).

This phenomenon of transient derepression is essential for the propagation of the plasmid and it is made possible by the activity of the FinO protein. After the plasmid DNA is tranferred to the recipient cell, it is first replicated. Almost immediately, transcription and translation of the various genes occurs. However, the absence of a FinO protein prior to the translation of the TraJ transcriptional activator allows the *tra* operon to be transcribed and further transfer events are initiated. Presumably, FinP is transcribed concurrently in the cell with TraJ mRNA, although it has no effect in the absence of FinO. This temporal separation of the fertility inhibition system from TraJ expression allows expression of TraJ and the *tra* operon until the FinO concentration in the cell reaches inhibitory levels and the pilus synthesis machinery is degraded or diluted out during cell division. This model suggests that it would be unfavourable for the plasmid to express a FinP RNA which is active in the absence of FinO. Such a plasmid would be deficient in post-mating transient derepression and would quickly be lost from a population of cells. Lundquist and Levin (1985) have proposed that the function of the conjugative repression system (fertility inhibition) is two-fold. First, repression of the synthesis of pili allows the population of donor cells to maintain a high level of resistance to infection by pilus-specific bacteriophages. Second, this system allows propagation of the plasmid throughout a population of cells in a transient but rapid manner.

The results presented in this thesis have been used to generate a model of FinOP-mediated inhibition of TraJ expression (Fig. 4i). In this model, the FinO protein is proposed to have a dual function, affecting both the stability of the FinP RNA, which is important *in vivo*, and also the rate at which the FinP-*traJ* mRNA duplex is formed, which has been demonstrated only *in vitro*. In the absence of FinO, FinP is expressed constitutively but it is quickly degraded by an unknown nuclease. As a result, the intracellular concentration of FinP is too low to mediate the inhibition of TraJ translation. However, in the presence of FinO, FinP is protected from RNase degradation by virtue of a direct interaction between FinO and stem-loop II of FinP. The subsequent increased size of the intracellular pool of FinP favours the interaction between FinP and *traJ* mRNA. FinO is also bound to the *traJ* mRNA and catalyzes the formation of the FinP-*traJ* mRNA duplex by at least 5-fold. The resulting stable duplex is likely to directly inhibit TraJ translation by either occlusion of the ribosome binding site or rapid degradation of the duplex by RNase III.

IV. Questions

There are a number of aspects of the FinO model which remain unclear. First, if the binding of FinO to FinP/*traJ* mRNA is specific, then what are the determinants required for specificity? The data presented in this thesis suggest that FinO interacts with a region of double-stranded RNA. Does FinO recognize a specific sequence or a sequence-specific conformational change in the helical nature of the RNA?

How does FinO promote the formation of the FinP-*traJ* mRNA duplex? It is reasonable to suggest that FinO acts in a Rom-like manner by inhibiting the rate of dissociation of the intermediate kissing complex. This type of interaction may involve protein-protein interactions between several molecules of bound FinO. By stabilizing the kissing intermediate, the local concentration of the free single-stranded tails of the RNAs may be increased, thereby favoring their interaction and subsequent nucleation. This activity of FinO may be required to help overcome the short single-stranded regions present in the FinP RNA. Also, it has not yet been determined which of the single-stranded regions, aside from the loops, of the FinP RNA, including the 5'- and 3'-tails, and the spacer, are important for stable duplex formation.

What is the nature of the interaction between FinO and the stem-loop II domain of FinP and *traJ* mRNA? The stem II domain of FinP is very unusual for a naturally occurring antisense RNA because it does not contain any disruptions in its double-stranded nature. Is FinO involved in unwinding the stem region, thereby allowing a deep-kissing intermediate to form? This may indeed be a significant possibility since the top of stem II is stabilized by the presence of three consecutive GC base pairs which would be expected to decrease the rate of duplex formation. Since the anti-RBS sequence is located in stem-loop I of FinP, it is possible that a duplex need not form in the stem-loop II region. Thus, a model which incorporates both duplex formation in the stem-loop I region and the formation of a stabilizing ribonucleoprotein complex at stem-loop II could also be used to explain the FinO binding data.

B. Conclusions

The characterization of the FinOP fertility inhibition system has yielded important new information about the mechanism of antisense RNA-mediated gene regulation and also the nature of protein-RNA interactions. FinO has been identified as a highly unusual protein which increases the chemical and functional stability of the FinP antisense RNA, and also mediates its interaction with the *traJ* mRNA. Its interaction with its duplex RNA binding site is unusual in that it appears to be specific for double-stranded RNA, thus the elucidation of the mechanism of this binding may provide valuable information on the nature of protein-RNA interaction.

Future experiments which should help identify the FinO binding site on FinP include identifying FinP mutations which directly affect FinO binding. Conversely, the isolation of mutants of FinO which are deficient in FinP/*traJ* mRNA binding should help identify the amino acids in FinO which are specifically required for formation of the FinO-FinP complex. Also, a series of *in vivo* experiments are required to identify the single-stranded regions of FinP required for stable duplex formation, and also to analyze the ability of stem-loop domains I and II to repress transfer independently of each other. The most significant hindrance to the analysis of the FinP-*traJ* mRNA interaction *in vivo* is the absolute requirement for FinO. Thus, it is important to identify the ribonuclease which mediates the rapid degradation of FinP in the absence of FinO. The FisP mutant may provide important clues to this degradation since it is less stable than FinP and it is unaffected by the presence of FinO.

It is hoped that the study of naturally occurring antisense RNA systems such as this one will facilitate the design of synthetic antisense molecules such as PNAs (peptide-nucleic acid chimeras) (Egholm *et al*, 1993), which can be used as tools to study the function of genes *in vivo* (Simons, 1993) and also for the specific treatment of genetic disorders (Neckers *et al*, 1992) and disease (Homann *et al*, 1993).

C. References

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